

STUDY OF MACROGLOBULINAEMIA  
IN TRYPANOSOMA EQUIPERDUM INFECTIONS OF THE RABBIT

by

J.M. ROSS

Submitted in fulfillment of the requirements

for the degree of M.Sc

in the

Faculty of Science

University of Cape Town.

The copyright of this thesis is held by the  
University of Cape Town.  
Reproduction of the whole or any part  
may be made for study purposes only, and  
not for publication.

September 1976

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

## ACKNOWLEDGEMENTS

I wish to express my sincere thanks to my promotor Professor M.H.V. Van Regenmortel, for his enthusiastic encouragement and guidance throughout the period of this study.

I also wish to thank Dr. A. Polson for his valuable advice, Miss K. Gill for her technical assistance, and Mr. D. Rossouw for his help with the experimental animals.

General assistance from other members of the department of Microbiology is also gratefully acknowledged.

This work was supported by a South African Medical Research Council grant awarded to Professor Van Regenmortel.

# ABBREVIATIONS.

BSA	Bovine Serum Albumin
DTT	Dithiothreitol
IE	Immunoelectrophoresis
NRS	Normal Rabbit Serum
PBS	Phosphate buffered saline
PBSGH	Phosphate buffered saline with glucose and Heparin
PEG	Polyethyleneglycol
SRBC	Sheep red blood cell
ZE	Zone electrophoresis

# CONTENTS

CHAPTER I	INTRODUCTION .....	1
CHAPTER II	LITERATURE SURVEY	
A.	General characteristics of Trypanosomes ...	4
1	The classification of trypanosomes .....	4
2	Morphology of trypomastigote form of trypanosomes .....	5
3	Life-cycle of trypanosomes .....	7
4	Host-parasite relationships .....	8
B.	Characteristics of <u>Trypanosoma equiperdum</u> .	8
1	Classification and distribution .....	8
2	Morphology and life-cycle .....	9
3	Host-parasite relationship .....	9
C.	Immunology of African and Animal Trypanosomiasis (Salivarian group) .....	10
1	Antigenic structure and variation of trypanosomes. ....	11
a	Characteristics of the antigenic structural groups .....	11
i	Variant antigens .....	11
ii	Exoantigens .....	12
iii	Common antigens .....	12
b	Antigenic variation .....	13
2	Immunization methods .....	15
3	Pathological effects of trypanosomes ...	16
4	Immunological response to trypanosome infections .....	17
a.	IgM and IgG immunoglobulin concentration. ....	17
b.	Quantitation of IgM .....	18
c.	Nature of the IgM .....	22
d.	Specificity of the IgM .....	24
i	Nature and occurrence of rheumatoid factors. ....	25
ii	Detection of rheumatoid factors .....	26
iii	Rheumatoid factors in trypanosomiasis .....	27
iv	Nature and occurrence of autoantibodies. ....	28
v	Autoantibodies in African trypanosomiasis .....	28
vi	Theories of the induction of autoimmunity .....	30
vii	Other IgM specificities .....	32
e.	Cell mediated response .....	33
f.	Suppression of the immune response..	33

## CHAPTER III MATERIALS AND METHODS

A. Buffers .....	36
B. Techniques with Trypanosomes .....	38
1 Maintenance and purification of trypanosomes.....	38
2 Activation of DEAE cellulose.....	40
3 Preparation of Trypanosome clones.....	40
4 Routine infection of rabbits with <u>T. equiperdum</u> .....	41
5 Inactivation of trypanosomes and immunization of rabbits .....	41
C. Purification of Rabbit IgM, IgG and Preparation of Antisera .....	42
1 Preparation of a Sephadex G200 column.....	42
2 Preparation of a zone electrophoresis column .....	43
3 Purification of rabbit IgM .....	46
a Euglobulin precipitation and gel filtration .....	46
b Zone electrophoresis .....	47
4 Reduction of 19S IgM .....	47
a Cysteine reduction .....	47
b Mercaptoethanol reduction .....	48
c Dithiothreitol reduction .....	48
d Purification of 7S and 13.4S IgM ....	48
5 Sucrose density gradient centrifugation.....	48
6 Preparation of goat antisera .....	49
a Preparation of goat anti rabbit IgM serum .....	49
b Absorption of goat anti rabbit IgM serum .....	49
c Preparation of goat anti NRS serum ...	50
d Preparation of goat anti rabbit IgG serum .....	50
7 Preparation of rabbit IgG .....	51
8 Immunoelectrophoresis .....	52
D. Quantitation of Rabbit IgM and IgG .....	53
1 Determination of IgM concentrations by quantitative gel precipitin tests.....	53
2 Laurell electrophoresis.....	56
3 Rocket electrophoresis .....	58
4 Analytical ultracentrifugation .....	58
a Calibration of the area under a schlieren peak by fringe shifts.....	58
b Determination of 19S concentrations in rabbit sera .....	59
c Determination of sedimentation coefficients .....	59
E. Methods for the Detection of Rheumatoid factors.....	62
1 Latex fixation .....	62
a The latex tube test .....	62
b The latex slide test .....	63
2 Passive haemagglutination .....	64

F	Methods for the Detection of Anti Trypanosome Antibodies .....	66
1	Indirect immunofluorescence .....	66
2	Direct immunofluorescence with rabbit IgM .....	68
3	Direct agglutination .....	70
G	Methods for the Detection of Autoimmune Antibodies .....	70
1	Micro-complement fixation .....	70
2	Absorption of sera with trypanosomes for micro complement fixation .....	75
3	Preparation of trypanosome antigens .....	75
4	Preparation of tissue antigens .....	75

#### CHAPTER IV RESULTS.

A	The purification of IgM .....	76
1	Purification of 19S IgM .....	76
a	Purification by euglobulin precipitation, gel filtration and zone electrophoresis .....	76
b	Purification of IgM from serum by polyethyleneglycol (PEG) precipitation .....	81
c	Purification of IgM from a gel filtration fraction by PEG precipitation .....	84
2	Purification of IgM <sub>5</sub> (monomeric IgM) .....	86
a	Cysteine reduction of IgM .....	86
b	Mercaptoethanol reduction of IgM .....	88
c	Dithiothreitol reduction of purified IgM .....	88
d	DTT reduction of the 19S IgM present in serum .....	91
3	Fractionation of serum by density gradient centrifugation .....	93
B	Production of Antisera .....	95
1	Preparation of goat anti rabbit IgM serum .....	95
a	Absorption of the antiserum with rabbit IgG .....	95
b	Absorption of the goat antiserum with rabbit neonatal serum .....	97
c	Absorption of the goat anti rabbit IgM serum with dialysed NRS .....	99
d	Attempts to isolate macroglobulin for absorption experiments .....	99
2	Preparation of goat anti rabbit serum ....	100
3	Preparation of goat anti rabbit IgG serum.	100

C	Quantitation of Rabbit IgM .....	100
1	Determination of IgM concentrations by quantitative immunoelectrophoresis .....	100
2	Determination of IgM concentrations by gel diffusion, ultracentrifugation and rocket IE .....	101
3	Attempts to quantitate IgM by Laurell electrophoresis .....	113
D	Quantitation of IgG in Rabbit Sera .....	116
E	Tests for rheumatoid Factors in Trypanosome Infected Rabbit Sera .....	119
F	The Nature and Quantitation of Anti Trypanosome Antibodies in the Sera of <u>T. equiperdum</u> infected Rabbits .....	125
G	The Nature and Quantitation of Tissue Autoantibodies In Trypanosome infected Rabbits .....	129
H	Immunization of Rabbits with ActinomycinD inactivated Trypanosomes .....	134

## CHAPTER V DISCUSSION AND CONCLUSIONS

A	Purification of IgM and the Production of Antisera .....	138
B	Immunoglobulin Levels .....	146
C	The Absence of Rheumatoid Factors (RF) .....	150
D	Anti-trypanosomal Activity of Immunoglobulins .....	155
E	Autoimmunity to Tissue Antigens .....	158

## CHAPTER VI BIBLIOGRAPHY .....

161



## SUMMARY

1. 19S IgM, 7S IgM, and IgG were purified from the serum of Trypanosoma equiperdum infected rabbits. The following antisera were also prepared for the quantitation of rabbit IgM and IgG:
  - i goat anti rabbit IgM antiserum
  - ii goat anti rabbit IgG antiserum
  - iii goat antiserum to rabbit serum proteins.
2. The serum levels of 19S IgM, measured by gel diffusion and rocket immunoelectrophoresis were shown to increase approximately ten times at the peak of the Trypanosoma equiperdum infections in the rabbit. The serum IgG concentrations, measured by gel diffusion increased more than seven times over the infection period. Low molecular weight 7S IgM was also detected in the serum by rocket immunoelectrophoresis and quantitative gel diffusion, but accurate concentration determinations could not be made.
3. Rheumatoid factors were not detected by passive haemagglutination and latex fixation tests, despite the use of a variety of sensitizing globulins. These results failed to confirm earlier reports of the presence of rheumatoid factors in infected rabbits

4. After separation of the 7S and 19S fractions by density gradient centrifugation, both fractions were found by complement fixation to contain anti trypanosome antibodies. These antibodies were also detected by immunofluorescence in the serum and 7S fractions, and by agglutination in the 19S fractions.
5. The 19S fractions also contained complement fixing antibodies reacting with the tissue antigens of rabbit liver, heart and kidney. Maximum titres of  $1/80 - 1/160$  were found at the peak of the infection. Absorption of the rabbit sera with trypanosomes indicated that the trypanosomal and tissue antibodies were distinct.

## INTRODUCTION.

Trypanosome infections of man and animals are characterised by a large and persistent increase in the level of serum IgM (Masseyeff and Lamy 1966, Houba et al 1969, Seed et al 1969), and in the production of autoantibodies (Mackenzie and Boreham 1974). The cause and biological significance of the raised IgM levels are still unknown, but it has been suggested that this persistent macroglobulinaemia is the result of sequential antigenic variation in the variant antigens of the parasites. Each successive antigenic variant could therefore, be expected to stimulate the production of new specific IgM antibody (Seed et al 1969). There is little evidence to support this hypothesis, as only a small proportion of the IgM has been shown to be specific for trypanosomal antigens (Masseyeff 1969).

Recently a low molecular weight variant of IgM (7S IgM) has been detected in the sera of patients with African trypanosomiasis, (Klein et al 1967) as well as in the sera of rabbits infected with T. equiperdum (Frommel et al 1970). Low molecular weight IgM has also been observed in normal human cord sera (Masseyeff et al 1972). Few studies on the IgM levels in experimental and African trypanosomiasis have, however, included a study of the low molecular weight IgM, and no accurate concentration determinations have been made.

The cause of 7S IgM production is still undetermined, but it is thought that low molecular weight IgM occurs as a result of the explosive IgM production (Frommel et al 1970), rather than as a result of IgM (19S) denaturation (Stobo and Tomasi 1967). The specificity and biological role of 7S IgM are still not known.

In addition to the low levels of anti-trypanosome antibodies found in African trypanosomiasis, the 19S IgM fraction has also been shown to contain heterophile antibodies and various autoimmune antibodies. The autoimmune antibodies in T. gambiense, T. brucei and T. congolense infections of rabbits, include antibodies to rabbit liver, heart, brain, spleen and kidney tissue, as well as to a fibrinogen component (Seed and Gam 1967, Mansfield and Kreier 1972, Boreham and Facer 1974, Mackenzie and Boreham 1974). No studies on the autoimmune response in T. equiperdum infections have been carried out.

One other group of autoantibodies of considerable interest are the rheumatoid factor-like substances which Klein and Mattern (1965), reported to be present in a small proportion of patients with trypanosomiasis. Their initial observations were later extended to experimental trypanosomiasis in the rabbit (Klein et al 1970) which led to the suggestion that the occurrence of rheumatoid factors in rabbits infected with Trypanosoma equiperdum might form the basis of a model for investigating the nature of rheumatoid factor formation (Klein et al 1970, 1971).

Rheumatoid factors have not been detected, however, in mice infected with T. gambiense, T. rhodesiense and T. brucei (Houba et al 1969, Klein et al 1971).

The research outlined in this thesis was undertaken to test the validity of the suggested model of rheumatoid factor induction in T. equiperdum of the rabbit, to measure the specificity of the 19S IgM and to establish whether there is an autoimmune response towards tissue antigens in the rabbits infected with Trypanosoma equiperdum.

## LITERATURE SURVEY

### A. GENERAL CHARACTERISTICS OF TRYPANOSOMES.

The recent understanding of mammalian trypanosomes owes much to investigations triggered by their role as causative agents of severe diseases of man and domestic animals.

These include

- 1) human trypanosomiasis - sleeping sickness in tropical Africa and Chaga's disease in the Americas, and
- 2) animal trypanosomiases - Nagana in tropical Africa, Surra in Africa North of the Sahara, tropical and sub-tropical Asia, central and South America, and Dourine, which has a cosmopolitan distribution.

### I. The Classification of Trypanosomes.

The systematic position of trypanosomes is as follows (Hoare 1972):-

<u>Phylum</u>	Protozoa
<u>Subphylum</u>	Sarcomastigophora
<u>Superclass</u>	Mastigophora
<u>Class</u>	Zoomastigophora
<u>Order</u>	Kinetoplastida
<u>Family</u>	Trypanosomatidae
<u>Genus</u>	Trypanosoma
<u>Sections</u>	a) Stercoraria
	b) Salivaria

- a) Stercoraria. This section comprises those species in which the developmental cycle in the insect vector is in the hindgut. Transmission is contaminative eg T. cruzi the causative agent of Chaga's disease.
- b) Salivaria comprising those species in which the development cycle in the insect vector is completed in the anterior portion of the gut. Transmission is inoculative. This section is subdivided into four sub genera.

- i Duttonella eg T vivax
- ii Nannomonas eg T congolense
- iii Pycnomonas eg T suis
- iv Trypanozoon eg T brucei, T gambiense,  
T rhodesiense, and T equiperdum.

The species of Trypanozoon are responsible for African trypanosomiasis and <sup>et al</sup> Dourine (Lumsden 1973).

In the genus Trypanosoma the following developmental stages are generally found:-

- i amastigote
- ii promastigote
- iii epimastigote
- iv sphaeromastigote, and
- v trypomastigote.

The trypomastigotes are the typical forms occurring in the blood of their vertebrate hosts.

## 2. Morphology of Trypomastigote Form of Trypanosomes.

Trypomastigote forms have an elongated body with a posteriorly placed kinetosome which is also known as the basal body of the flagellum. (Fig. 1).

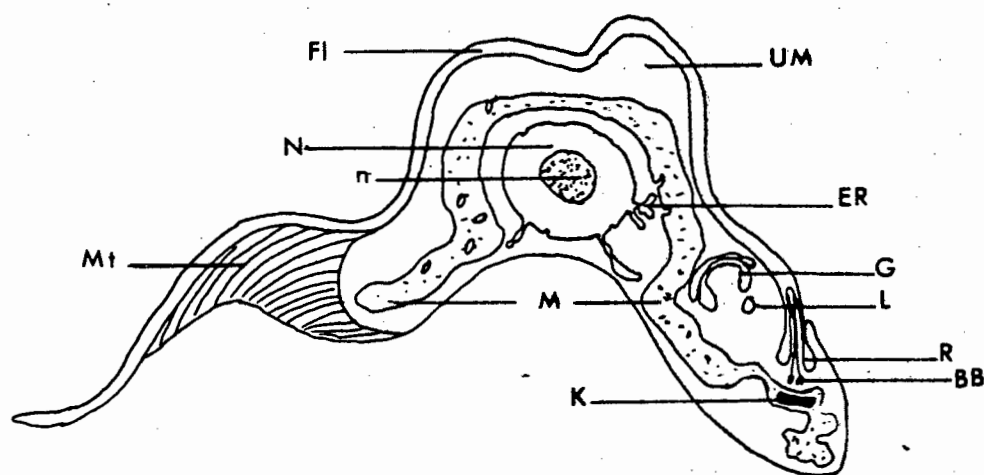


Fig. 1. Diagram of the ultrastructure of the trypomastigote form *Trypanosoma brucei*. Fl, flagellum; UM, undulating membrane; ER endoplasmic reticulum; G, Golgi apparatus; L, lysosome; R, reservoir; BB basal body or kinetosome; K, kinetoplast; M, mitochondrion; Mt, microtubules; n, nucleolus; N, nucleus. The trypomastigote form has a cell volume of  $50 \mu\text{m}^3$ . (White 1973).



This is partly intracellular and partly extracellular, since it projects into the reservoir from which the flagellum emerges. The flagellum is attached to the trypanosome along its length by a physical association between the flagellar membrane and the cell membrane. A system of microtubules lies beneath the surface membrane and extends from one end to the other parallel to the surface. Adjacent to the basal body is the kinetoplast, a DNA containing structure, sometimes regarded as "Mother mitochondrion" since it contains the genes for mitochondrial enzyme synthesis. Other cytoplasmic structures eg Golgi apparatus, lysosomes, ribosomes and endoplasmic reticulum are similar to those of mammalian cells.

### 3. Life Cycles of Trypanosomes.

The life cycles of trypanosome species vary considerably. Most have one stage in a vertebrate and another in an invertebrate host, although some species are restricted to either vertebrates or invertebrates. There is a striking difference between *Stercoraria* and *Salivarian* species in the multiplication pattern. In the *Stercoraria* multiplication is discontinuous, being limited to a reproductive period or occurring periodically. The *Salivaria*, on the other hand, have no special reproductive period, and multiply more or less continuously throughout the infection.

#### 4. Host - Parasite Relationships.

Trypanosomes infecting mammals can be divided into pathogenic and non-pathogenic groups according to whether or not the infection produces recognizable disease and pathological changes in the host. Most of the Stercorarian species, with the exception of T. cruzi are non-pathogenic, whereas Salivarian species are all potentially pathogenic to man and domestic animals. Since pathogenicity indicates poor adaptation between host and parasite and suggests that their association is recent, it is thought that the Stercorarian group is phylogenetically older than the Salivarian group. This idea is substantiated by the fact that Stercorarian species have a highly restricted host range and adapt with difficulty to foreign hosts.

#### B. CHARACTERISTICS OF TRYPANOSOMA EQUIPERDUM.

As the work outlined in this thesis was carried out using T. equiperdum a discussion of its essential characteristics follows.

##### 1. Classification and Distribution.

T. equiperdum belongs to the subgenus Trypanozoon of the group Salivaria and is the causative agent of the venereal disease of equines, or Dourine, which was first described by a Byzantine veterinarian in AD 400.

Dourine was well-known to horsemen of Africa at this time, but it was introduced to Europe and elsewhere much later with the spread of imported equines. Thus with the introduction of Persian stallions to Russia, and Syrian stallions to France, Dourine was introduced into Europe. The disease is now also prevalent in South Africa, South West Africa, Botswana, and in South America in Chile, Venezuela and Brazil.

## 2. Morphology and Life Cycle.

The structure of T equiperdum is similar to that already described apart from the fact that the trypomastigotes of this species are more slender and possess a long free flagellum. T equiperdum also has a tendency to produce dystkinetoplastic strains, ie. strains in which the kinetoplast has lost its DNA.

T equiperdum has no invertebrate host and therefore no cyclic development. The trypanosome is normally transmitted by coitus and reproduces within its host by binary fission. T equiperdum differs further from other mammalian trypanosomes in that it is primarily a tissue parasite, which rarely invades the bloodstream, only doing so en route to infect fresh tissues.

## 3. Host - Parasite Relationship.

The trypanosomes develop in the genital tract of the recipient horse immediately after their transmission, although the first symptoms only appear up to four months later. Oedematous patches first appear in the genital tract, and later after the parasites have passed into the bloodstream, urticarial plaques appear in the skin in various parts of the body. The trypanosomes then proliferate in the plaques and remain in the subcutaneous tissues. The later stages of Dourine are characterized by anaemia and nervous disorders, chiefly paralysis in the hind limbs. The duration of the disease varies from a few months to five years.

Since T. equiperdum is primarily a tissue parasite its establishment in the blood of laboratory animals presents much difficulty. Murine rodents can be infected from horses only after hundreds of unsuccessful attempts or by first inoculating splenectomized animals. Once the strain becomes adapted to these animals, however, it can be passaged for indefinite periods. Trypanosomes first appear in the blood of mice after two days, and death occurs after three to four days. Rabbits, however, manifest typical Dourine symptoms after two weeks, and die four to five weeks after inoculation. Attempts to infect domestic ruminants with T. equiperdum produces low or inapparent parasitaemia, while in pigs the infection is latent. No cases of human infection with T. equiperdum have been reported.

#### C. IMMUNOLOGY OF AFRICAN ANIMAL TRYPANOSOMIASIS (SALIVARIAN GROUP).

Interest in the immunology of trypanosomiasis first developed at the beginning of the twentieth century when many of the basic principles of immunology were being elucidated. The immunology of trypanosomes is difficult to study for the following reasons:

1. There is little natural immunity,
2. Recovery from virulent infections is rare.
3. There is great multiplicity of trypanosome species, and
4. considerable antigenic variation exists within a species.

Extensive research is at present being carried out on the immunological aspects listed below, with a view to improving understanding of the course of the disease and facilitating the diagnosis. The ultimate aim is prevention of trypanosomiasis.

This was produced by the Golgi derived reticulum of the trypanosomes. (Steiger 1973). Since the coat proteins prepared from different clones of one strain of *T. brucei* differed in composition, it now seems likely that the coat protein itself is the variant antigen. Furthermore, immunizing mice with the purified coat glycoprotein leads to protection against subsequent infections with living trypanosomes of the same clone (Cross 1975).

- (ii) The group of exoantigens includes those antigens not attached to the cell, i.e. soluble or free antigens. These antigens have properties similar to those <sup>of</sup> variant antigens, and in 1971 Allsopp et al provided evidence that variant antigens and exoantigens are chemically identical. Since it can be argued that soluble or exoantigens may only occur in vitro as a result of disintegration of the parasites and may not be present in vivo, it may be preferable to group the variant antigens and exoantigens together.
- (iii) Common antigens are those antigens within the cell, consisting of cytoplasmic and nuclear components. They appear to remain unchanged during the course of the infection, and are released at the peak of each parasitaemic wave. Common antigens elicit antibodies detectable by complement fixation, agglutination and immunofluorescence, and are as a rule typical of, but not specific for each species.

b) Antigenic Variation.

Although it is well-known that antigenically distinct trypanosome variants appear in succession during an infection, a feature which is typical of trypanosoma species, little is known concerning the cellular control of this antigenic variability.

Cantrell in 1958 suggested a random mutation mechanism, taking place independently of external stimuli. The main objections to this theory are that for a given strain of trypanosomes, antigenic variation occurs according to a predictable pattern (Gray 1962) and that there is frequent reversion to the basic antigenic type. (Gray 1965).

Gray in 1965, proposed a cell-organised process of antigenic variation, which would, of course, explain any regularity in the production of variants.

However this did not explain why only a few trypanosomes in each population are capable of using a genetically controlled device to escape the defence mechanism of the host. Another complication in this process was the finding by McNeillage et al (1969) that after single organisms have been inoculated into an animal the first relapse population contained more than one antigenic type at the same time.

If different variants are present simultaneously in the host, why is there a clear-cut interval between episodes of parasitaemia?

De Raadt (1974) suggests that the explanation depends on biological competitiveness: If one accepts that each strain of trypanosome has a certain spectrum of variants, then it is possible that, at any one time, one variant predominates over the others. The latter variant is suppressed, until the host has removed the virulent variety. At this stage, the next most virulent variety may become dominant, resulting in the next wave of parasitaemia. In this way the various antigenic types would appear in the order of virulence. This is consistent with the "regularity" of the variation cycle, with the occasional occurrence of a stable or parent antigenic form and with the anticipated omission of a variant in animals immunized against that variant. De Raadt further suggests that virulence is coupled with random mutation although it is generally felt by other authors that the spectrum of variants is too well-defined to have been produced as a result of random mutation.

Desowitz (1970) has proposed that antigenic variation could result from a cell-independent immunochemical reaction taking place at the cell surface, as a part of the antigen-antibody reaction. This theory is currently favoured (Cross 1975) despite the difficulty of explaining the occurrence of mixed populations of trypanosome variants.

To date most of the hypotheses are supported by inconclusive evidence, and the control of antigenic variation remains an enigma.

## 2. Immunization Methods.

Natural immunity to trypanosomes is essentially dependent upon the specificity of the host-parasite relationships, and as such is of limited importance in the course of infections of a parasite in its natural host. There are, however, cases of human carriers of sleeping sickness in whom natural immunity must occur, and similarly there are certain animal hosts naturally resistant to infection, eg baboons are immune to all pathogenic trypanosomes (Hoare 1972).

Attempts to produce immunity in animals have included both active and passive immunization methods. Active immunization was first carried out with limited success by injecting animals with dead or formol-inactivated trypanosomes (Soltys 1964). Gamma irradiation of parasites, which interferes with their multiplication, has proved to be more successful (Duxbury and Sadun 1969, Duxbury et al 1972). Other trypanosome inactivation methods have included Actinomycin D inactivation (Fernandes et al 1965,) and Chagastoxin treatment of trypanosomes (Seneca et al 1966).

A high level of general immunity has not yet been achieved probably due to antigenic variation of trypanosomes, multiplicity of trypanosome species and variant specificity of protective anti-trypanosome antibodies. The problem of antigenic variation could explain the unsuccessful immunization attempts made using dead trypanosomes as the immunogen, and the greater success using injections of live trypanosomes followed by treatment of the animal with trypanocidal drugs. (Soltys 1955, Whiteside 1962). The latter immunization

attempts/....



attempts, although successful in the laboratory, would be unsuitable for general immunization campaigns, because of the possibility of a widespread dissemination of drug resistance in trypanosoma species.

Passive immunization has been carried out successfully in laboratory animals by immunization with either spleen cells from immune donors (Takayanagi<sup>et al</sup> 1969, Viens et al 1974), thymic cells (Takayanagi and Nakatake 1976), or immune sera (Seed and Gam 1966, Takayanagi 1971).

### 3. Pathological Effects of Trypanosomes.

Despite the medical importance of trypanosome infections, little is known on the mechanisms of its pathogenesis - even the cause of death is still not known. Many of the studies on the pathological effects of trypanosomes have been carried out on T. brucei, a species which lives in the blood, lymph, cerebrospinal fluid, brain and connective tissues of the infected animal. Although their movement is unceasing there is no evidence that the activity of the trypanosomes is in itself damaging to the tissues of the host. However the demand for energy and the activation of the immune response involving plasmin formation, the release of kinins (Boreham 1968) and the deposition of immune complexes are all potentially harmful (Goodwin 1974). Furthermore, there is a marked suppression of cell mediated immune responses resulting in increased susceptibility to bacterial and viral infections.

Goodwin et al (1973), have shown an increase in pyruvate concentrations in the tissue fluids, which is associated with changes in the connective tissue such that the fibroblasts become laden with lipid droplets and cease to produce collagen fibres. Skeletal and heart muscle have also been found to

degenerate in T brucei infected animals. In the T gambiense infected rabbits, an elevation in the total serum lipids has been observed (Diehl and Risby 1974). Another major contributory cause of death in T brucei infections of murines appears to be hypoglycaemia (Herbert et al 1975). The possible damage caused by the host's immune reaction to the trypanosomes will be outlined in the following sections.

Work on the pathological effects of trypanosomiasis has also been carried out on T congolense, a species typical of those living entirely in the circulatory system. In this case accumulation of trypanosomes in the cerebral blood vessels and blood vessels of other selected organs, and anaemia appeared to be the main pathogenic factors. (Losos et al 1973).

#### 4. Immunological Response to Trypanosome Infections.

The immunological phenomena that occur in man and animals on infection with those trypanosomes belonging to the sub-genus Trypanozoon, have been the subject of many papers and an extensive review of the immunological aspects of trypanosomiasis will be presented in the following section.

##### a) IgM and IgG Immunoglobulin Concentrations.

The most characteristic feature of the humoral response is the prolonged increase in the concentration of IgM in the sera of trypanosome infected patients; This was first observed by Mattern et al (1961). Confirmation of the increased IgM levels was made in 1966 by Masseyeff and Lamy using the technique of single radial immunodiffusion. The IgM levels reached a maximum of five times that of the normal controls, and IgG level one and a half times

that of .... /

that of the normal controls while the IgA remained normal or was slightly reduced. The IgM concentration has also been reported to be raised in the C.S.F. of patients with either T rhodesiense or T gambiense infections (Mattern 1964, Mattern et al 1967, Goidl and Marsden 1974).

Similarly elevated IgM levels have been found in mice infected with T. gambiense by the techniques of immunoelectrophoresis, double diffusion and radial immunodiffusion. (Takayanagi and Enriquez 1973, Capbern et al 1974), and in calves infected with T vivax (Clarkson et al 1975).

In all these trypanosome infections the IgG concentration was also increased, the titre rising almost simultaneously with the IgM increase.

Seed et al (1969) infected rabbits with T gambiense and observed IgM levels four to eight times the normal level, eight days after infection, and an eight to sixteen fold increase by eighteen days. The IgM then reached a plateau and remained at this level for as long as one hundred and seventeen days. Determinations of the IgM concentration were made by double diffusion against goat-rabbit IgM serum.

Rabbits infected with T equiperdum also exhibit similar IgM patterns (Klein et al 1970). The IgM increased in concentration from a basal 1 mg/ml to a maximum of 8.6 - 11.3 mg/ml at day 25. Indirect determinations of the IgM concentration were made, however by subtracting the  $\alpha_2$  macroglobulin content from the total macroglobulin calculated from the 19S Schlieren peaks. IgG determined by radial immunodiffusion was increased from 10 mg/ml to 43 mg/ml at day 25, the increase occurring slightly later in the infection period than that of the IgM. The increase in IgM concentration was correlated with the appearance of rheumatoid factors.

Several hypotheses have been put forward to account for the prolonged increase in IgM concentrations. First the continual antigenic variation of the trypanosomes during the course of the infection could result in the synthesis of new IgM antibodies specific to each successive antigenic variant (Seed et al 1969, Capbern et al 1974). This, however, cannot be the only explanation, since little of the IgM appears to be directed towards the variant antigens of the trypanosomes (Masseyeff 1969). Another hypothesis is the blocking of the IgM to IgG switching mechanism, perhaps by a trypanosome-induced inhibitor (Goidl and Marsden 1974) or by defective B cell control (Terry et al 1973).

The pathological importance of the raised IgM concentrations is not known although it has been suggested that since they are large molecular weight proteins, they may upset the osmotic balance within the circulation, contribute towards an increased blood viscosity and cause an increased erythrocyte sedimentation rate. (Boreham and Facer 1974).

b. Quantitation of IgM

Quantitation of IgM in the sera from trypanosome infected humans or animals has been carried out by either serological techniques such as radial immunodiffusion (Masseyeff and Lamy 1966, Klein et al 1970, Capbern et al 1974) and double diffusion (Mattern et al 1961, Seed et al 1969) or by biophysical techniques such as analytical ultracentrifugation (Mattern et al 1967, Klein et al 1970).

In radial immunodiffusion, antiserum to the IgM is incorporated into the agar, and diffusion of the IgM occurs radially from the antigen well.

Klein et al (1970) reported difficulty with this technique since by plotting the square of the diameter of the precipitin ring against serum dilution of the various rabbit sera, the lines did not intersect the vertical axis at the same point. Such problems were not reported by Masseyeff and Lamy (1966), Capbern et al (1974).

The alternative serological method - double diffusion in agar is a technique whereby twofold dilutions of the test sera and a suitable dilution of anti-IgM serum diffuse from two parallel rows of wells. Precipitin bands are formed and the IgM titre is the last twofold dilution of the test serum to form a precipitin band with the antiserum.

IgM concentrations have also been determined in the analytical ultracentrifuge by measuring the area of Schlieren peaks corresponding to 19S components (Mattern et al 1967), and correcting for the presence of  $\alpha_2$  macroglobulin (Klein et al 1970).

The antisera that have been used for the quantitation of human IgM were in most cases prepared in horses, (Mattern et al 1961, Masseyeff and Lamy 1966), while for mouse IgM the antisera were prepared in rabbits (Takayanagi and Enriquez 1973). Anti rabbit IgM sera were prepared in goats (Seed et al 1969) or sheep (Klein et al 1970). IgM used for immunization was purified by a combination of gel filtration and either starch block electrophoresis (Klein et al 1970), or sucrose density gradient centrifugation (Seed et al 1969). IgM antisera were absorbed with purified rabbit  $\alpha_2$  macroglobulin and  $\beta$  lipoprotein.

Considerable difficulty is experienced in the purification of rabbit IgM owing to contamination with  $\alpha_2$  macroglobulin.  $\alpha_2$  macroglobulin is similar to IgM

in size.../

in size, and in neutral salt solubility. Removal of this contaminant requires exhaustive gel filtration or a combination of gel filtration and preparative electrophoretic techniques.  $\alpha_2$ macroglobulin is highly antigenic and complete removal is necessary for the preparation of a  $\mu$ chain specific antiserum. Absorption of antibodies to  $\alpha_2$ macroglobulin from an IgM antiserum is equally difficult since it requires a purified  $\alpha_2$ macroglobulin preparation.

c. Nature of the IgM.

When serum IgM concentrations were determined by immunodiffusion, the values were higher than when determined by ultracentrifugation (Mattern et al 1967, Solomon 1969). Klein et al (1967) proposed that this discrepancy was due to the fact that some of the sera contained a low molecular weight IgM in addition to the pentameric form. Confirmation of this proposal was made by Jones (1970), who detected discrepancies between serum IgM concentrations determined by immunoelectrophoresis and immunodiffusion. Monomeric 7S IgM was first found in patients with lupus erythematosus (Rothfield et al 1965), and since then it has been found in patients suffering from the following diseases:- malignancy of plasma cell or lymphatic systems (Solomon and Kunkel 1967, Solomon 1969, Dammacco et al 1970), type I dysgammaglobulinaemia (Gleich et al 1966), Waldenstrom's macroglobulinaemia, (Stobo and Tomasi 1967, Bush et al 1969, Eskeland and Harboe 1973), rheumatoid arthritis (Stage and Mannik 1971, Los Palluto 1968) and trypanosomiasis (Klein et al 1967, Frommel et al 1970, Masseyeff et al 1972).

7S IgM has also been observed in normal human cord sera (Perchalski et al 1968, Starța - Geribaldi and Masseyeff 1975), and in an extremely low proportion of normal African adult sera (Masseyeff et al 1972) and even in horse serum (Sándor et al 1964).

This naturally occurring low molecular weight IgM ( $\text{IgM}_N$ ) is similar in size to in vitro produced monomers ( $\text{IgM}_S$ ) and appears to have the same antigenic determinants when tested by double diffusion (Stobo and Tomasi 1967, Swedlund et al 1968, Masseyeff et al 1972). It does, however, have a larger negative charge than  $\text{IgM}_S$  or pentameric IgM, and this results in anodal extensions of the IgM immunoelectrophoretic precipitin lines (Hansson and Laurell 1970; Masseyeff et al 1972).

Methods for the detection and quantitation of  $\text{IgM}_N$  have varied from double diffusion in 4% acrylamide gel (Stobo and Tomasi 1967), or 3.5% acrylamide plus 0.8% agarose (Masseyeff et al 1972), to the separation of 19S and 7S IgM by sucrose density gradient centrifugation or gel filtration followed by radial immunodiffusion against an anti-IgM serum (Bush et al 1969). An alternative separation method based on differential precipitation with polyethyleneglycol has recently been developed (Howba and Lambert 1974).



Attempts to quantitate the  $\text{IgM}_N$  have been made in at least two diseases - Waldenstrom's macroglobulinaemia and experimental trypanosomiasis in rabbits. In the former case  $\text{IgM}_N$  appeared to range from 10 - 45% of the total IgM (Stobo and Tomasi 1967, Bush et al 1969), whereas in the latter case satisfactory quantitation was not achieved. Estimates of  $\text{IgM}_N$  content were, however, made at approximately 5% of the total IgM in the trypanosome sera (Frommel et al 1970).

Neither the significance of this  $\text{IgM}_N$ , nor the reasons for its production are known.  $\text{IgM}_N$  does not appear to form by in vitro degradation (Stobo and Tomasi 1967), although this has not yet been excluded. It is possible that owing to explosive production of IgM in the diseased states mentioned,  $\text{IgM}_N$  increases as a result of incomplete 19S IgM formation from a normal level below the threshold of detection to one easily detectable. This idea is supported by the recent finding of free light chains in the cerebrospinal fluid of patients with trypanosomiasis (Greenwood and Whittle 1975).

d) Specificity of the IgM

Various authors have reported that the IgM in sera from trypanosome-infected organisms is directed against IgG, tissue antigens and trypanosomes.

(i) Nature and Occurrence of Rheumatoid Factors.

Rheumatoid factors are antibodies with reactivity for the rheumatoid arthritis patient's own gamma globulins, and were first detected by Cecil et al in 1931. Further studies by Waaler in 1940, and Rose et al in 1948 indicated that sheep erythrocytes sensitized with sub-agglutinating dilutions of rabbit IgG antibody to sheep red blood cells were agglutinated by anti-gamma globulins found in the sera of rheumatoid arthritis patients. Subsequent studies by Franklin et al in 1957 showed the formation of 22S complexes between 19S IgM rheumatoid factors and autologous 7S IgG, thus indicating the IgM nature of the rheumatoid factors. More recently it has been shown that rheumatoid factors are also found in the IgA and IgG subclasses (Harboe et al 1965, Lyst and Normansell 1974).

Human rheumatoid factors have a broad degree of reactivity for IgG from humans and rabbits and other animals (Winblad et al 1969). Many rheumatoid factors react preferentially with antigen - antibody complexes, and aggregated or denatured IgG, although reactivity with native autologous IgG to form 22S complexes also occurs. (Vaughan 1956, Edelman et al 1958, Singer et al 1960, Bernhard et al 1962, Normansell 1970, Lindström 1973). The vast majority of rheumatoid factors show optimal reactivity with the Fc portion of IgG molecules (Gaarder and Natvig 1970).

Although rheumatoid factors are common in patients with rheumatoid arthritis they are by no means found only in this disease. Rheumatoid factors also occur in subacute bacterial endocarditis, lupus erythematosus, chronic infectious diseases and African trypanosomiasis. (Panush 1974, Houba and Allison 1966).

There is as yet no firm evidence as to the precise role played by rheumatoid factors in the body. Both IgG and IgM rheumatoid factors can activate the classical complement system and in rheumatoid arthritis cases intra-articular activation is known to occur. (Williams 1974). Rheumatoid factors may also enhance the interaction of certain immune complexes with phagocytic cells.

#### ii Detection of Rheumatoid Factors.

The most commonly used tests for the detection of rheumatoid factors are sensitized sheep cell agglutination, Waaler-Rose tests and latex fixation tests. In the sheep cell test, sheep cells are sensitized with sub-agglutinating amounts of antibody and then reacted with test sera. In the Waaler-Rose test human erythrocytes are sensitized with rabbit IgG antibodies, while in the latex fixation test, latex particles are coated with human IgG. The latex test is the easiest to perform and the most sensitive but is subject to considerable non-specific agglutination by substances other than rheumatoid factors. (Watson 1965, Griebble et al 1969).

Some sera contain thermolabile non-specific agglutinators, (eg Clq a portion of the first component of the complement system), and must therefore be heat inactivated prior to testing (Cheng and Persellin 1971). For the detection of human rheumatoid factors, rabbit IgG is commonly used for the sensitization of latex particles (Cheng and Persellin 1973).

Both the Waaler-Rose test and the sensitized sheep cell test are more specific for rheumatoid factors than the latex test (Waaler, 1940. Holley et al 1961. Cheng and Persellin 1973), but they are time consuming and less sensitive (Grieble et al 1969).

### iii Rheumatoid factors in Trypanosomiasis

The presence of rheumatoid factors in human sera was first described in patients suffering from T gambiense infections (Klein and Mattern, 1965). Houba and Allison (1966) confirmed the presence of rheumatoid factors in cases of T. gambiense and T rhodesiense infections, and in 1970 Klein et al (1970) reported the detection, by latex fixation and Waaler-Rose tests, of rheumatoid factors in rabbits experimentally infected with T equiperdum. These authors suggested that the high frequency of rheumatoid factors in these animals might form the basis of a model for investigating the nature of rheumatoid factor formation. Rheumatoid factors have not been detected in mice infected with T. gambiense, T equiperdum nor in monkeys infected with T gambiense, T rhodesiense and T brucei (Klein et al 1970, 1971).

iv Nature and Occurrence of Autoantibodies.

Autoantibodies are "antibodies capable of specific reaction with an antigen that is a normal constituent of the body of the individual by which the antibodies were formed" (Herbert and Wilkinson 1971). When such antibodies were first discovered they were thought to occur rarely and only with disastrous consequences. However, with increasingly sensitive techniques it has become obvious that autoantibodies are quite common particularly in connective tissue diseases such as rheumatoid arthritis, and that they even occur at a very low titre in normal individuals. The pathological effects of autoantibodies, however, are still conjectural. Tissue autoantibodies have also been described in a wide variety of infectious diseases including yellow fever (Hughes 1933), syphilis, malaria (Davis 1944), schistosomiasis (Bassily et al 1973), and African trypanosomiasis (Mackenzie and Boreham 1974, Mansfield and Kreier 1972a, Seed and Gam 1967).

v Autoantibodies in African Trypanosomiasis.

Anti - tissue antibodies were first described in 1961 by Muschel et al in T gambiense and T rhodesiense infections of rabbits. In 1967 Seed and Gam used complement fixation tests to detect anti-liver autoantibodies in T gambiense infections of rabbits. These antibodies reached a peak eighteen days after infection with a maximum titre of  $1/512$  and then slowly decreased. Pre-infection titres of  $1/8$  were considered negative because of the presence of anti-complementary activity at low dilutions.

Natural antibodies to a liver antigen are commonly found in normal rabbits older than one month, although the titres are not high and range from  $\frac{1}{2}$  to  $\frac{1}{16}$ . (Kidd and Friedewald 1942, Mackenzie and Boreham 1974).

Mansfield and Kreier (1972a) made a study of autoimmunity to tissue antigens in T congolense infected rabbits. They studied both autoantibody production and evidence for cell mediated immunity (See section C, 4, e) and demonstrated the presence of complement fixing and precipitating auto-antibodies reacting with rabbit heart, brain, liver and kidneys. The largest antibody response was directed against brain antigens. Wassermann antibodies were not detected, and no autoantibodies were found in the normal controls. Physico chemical analyses of the autoantibody indicated that it was exclusively IgM. This result was confirmed in 1974 by Mackenzie and Boreham and distinguishes this type of response from the rabbit anti - liver autoantibodies of IgG type produced by immunization with liver extracts (Asherson and Dumonde 1962, 1964).

Rabbits infected with T brucei, however, showed a substantial increase in the natural anti-liver autoantibodies and Wassermann antibodies with titres reaching a maximum of  $\frac{1}{512}$  (Mackenzie and Boreman 1974). Absorptions indicated that the anti-liver and Wassermann antibodies were distinct. Furthermore since both autologous and uninfected homologous liver antigens gave similar complement fixation results, it was deduced that the effective antigen was not of trypanosomal nature. This was confirmed by absorption studies (Mackenzie and Boreham 1974, Mansfield and Kreier 1972a).

While attempting to measure the concentration of fibrin, and fibrinogen degradation products in the sera of rabbits infected with T. brucei, Boreham and Facer (1974) observed the presence of an autoantibody to a fibrinogen component. The development of autoantibody was identical to that of anti-liver antibodies, maximum titres of  $1/512$  being reached one to two weeks after infection. Low levels of the same autoantibody were found in normal control sera. The nature of the substance acting on the antigen is not known but the authors suggested that it could be either a degradation product of fibrinogen or a fibrinogen molecule altered, such that hidden antigenic determinants are exposed. It is possible that during the polymerization of fibrinogen to fibrin the same antigenic sites are exposed since absorption of serum with autologous fibrin removes most of the antibody activity.

#### vi Theories of the Induction of Autoimmunity.

Several theories of the induction of autoimmunity during infectious diseases have been formulated. The possible existence of antigenic determinants shared by trypanosomes and host tissues has been mentioned although this is unlikely in view of the results of absorption studies (Mackenzie and Boreham 1974). Secondly minor tissue damage caused by the trypanosomes may result in the release of tissue antigens, but this was disputed by Mansfield and Kreier in 1972(a), as in T. congolense infections, auto-antibodies arose well before any observable tissue necrosis.

However, since extensive kidney damage was observed during microscopic examinations of the tissues, it is quite possible that an antigen which is not specific for the kidney would be released at the onset of kidney damage. Thus autoantibodies directed against released kidney antigens could cross-react with liver and heart antigens. In fact Mackenzie and Boreham (1974) absorbed sera with kidney antigen and removed the anti-liver activity.

Another possibility is the action of trypanosomes as a general adjuvant. This would not operate through the T-cell system as appears to occur with Freund's complete adjuvant (Allison et al 1971), owing to the marked B-cell response found in trypanosomiasis. Mansfield and Kreier (1972a) also suggest that a thymic malfunction may allow B cells to escape from a T-cell induced control system: this would allow the normally low levels of liver and Wassermann antibodies to rise.

Interest in the autoantibodies occurring in trypanosome infections has increased because the cause of death in domestic animals and man is still uncertain, and such autoantibodies could play an important part in the pathogenesis of the disease. However, the contribution of anti-liver autoantibodies to the pathology in trypanosomiasis appears to be slight since the antibody is present in normal rabbits at low levels, and apparently does no harm. In addition the serum transferase levels of infected rabbits suggest no gross liver damage.

(Mackenzie and Boreham 1974)



Similarly low levels of anti-fibrin autoantibody exist normally in rabbits, and high concentrations found in infected sera do not induce hypofibrinogenaemia (Boreham and Facer 1974).

Autoantibodies may, on the other hand, produce "inapparent" pathological changes, such as the blocking of membrane sites which are important enzyme recognition sites, or the coating of certain tissues to prevent adequate nutrient and gas exchange with the blood (Mansfield and Kreier 1972a). Apart from these possibilities, auto-antibodies contribute to the raised IgM content of the sera which is likely to lead to an increased viscosity of the serum.

#### vii Other IgM Specificities.

Other antigens to which the IgM antibodies have been shown to be directed include the variant antigens of the trypanosomes (Mattern et al 1961, Seed et al 1969, Takayanagi and Enriquez 1973), and Forssman antigens i.e. heterophile antibodies (Mackenzie and Boreham 1974, Houba et al 1969).

#### e. Cell Mediated Response.

There is little substantial evidence that cell mediated immunity occurs in trypanosome infections apart from the demonstration of skin hypersensitivity in rabbits infected with T. brucei (Tizard and Soltys 1971).

Cell mediated hypersensitivity to trypanosome antigens was absent in T congolense infections of rabbits (Mansfield and Kreier 1972b) and in T rhodesiense infections of man in which lysed trypanosomes were injected (De Raadt et al 1967). However, this absence may have resulted from the relative absence of tissue invasiveness exhibited by T congolense and T rhodesiense. Cell mediated hypersensitivity to brain and other tissue antigens in T congolense infections was also absent as shown by negative skin tests, migration inhibitory factor and skin reactive factor tests, (Mansfield and Kreier 1972a).

f. Suppression of the Immune Response.

It is well established that T brucei infections in rodents have an immunosuppressive effect on the response to sheep red blood cells (Goodwin 1970, Murray et al 1973, Goodwin et al 1972). Murray et al (1974b) attempted to determine whether the immunosuppression associated with trypanosomiasis was caused by a defect in the function of T or B lymphocytes. Results indicated a failure in antibody production at the cellular level i.e. a B cell defect. T cells appeared relatively normal as judged by their response to oxazolone sensitization. This is in direct contrast to Mansfield and Wallace (1974) who infected rabbits with T congolense and tested for in vivo and in vitro cell mediated responses. Skin test responses to a purified protein derivative were less marked in infected, immunized rabbits than in control rabbits. Similarly the responses of the peripheral blood.../

blood lymphocytes to the protein derivative were lower than in infected animals, thus indicating a suppression in the cell mediated immune system. The mechanism by which trypanosome infections cause this apparent suppression is not known, although possibilities include the occurrence of a serum suppressive factor, selective T cell depletion, or a defect at the level of the macrophage cell contributing to T cell deficiency in infected animals. (Mansfield and Wallace 1974).

This appears to be unlikely in view of the fact that clear evidence of macrophage dysfunction was not obtained by Murray et al (1974a) in their experiments with T brucei infections.

It therefore appears that the T cell failure may occur particularly in the terminal stages of the disease, the extent being dependent upon the nature of the infecting organism, and presumably on the general condition of the patient or animal. Since evidence for B cell defectiveness has been reported only in the case of T brucei infections of mice (Murray et al 1974b) one can only presume that the occurrence is relatively infrequent and perhaps dependent upon the anaemic condition of the animal. It would be expected that if immune suppression were significant more patients with sleeping sickness would manifest other infectious diseases, and in fact De Raadt (1974) notes that resistance to concurrent infections seems stronger rather than diminished.

An attempt to explain the apparent paradox of heightened immunological activity accompanied by the possible immunosuppression in trypanosomiasis was made by Terry et al (1973). They suggest that trypanosome infections break the control link between thymus dependent lymphocytes and thymus independent lymphocytes. Escape from control might allow the B cells to proceed with the manufacture of IgM antibodies, whilst other responses requiring cellular interactions would be suppressed.

Another more realistic hypothesis for explaining the abnormal immune response is that of the occurrence of a B cell mitogen in trypanosomiasis (Urquhart et al 1973, Greenwood 1974). It is possible that trypanosomes produce a B cell mitogen which has a non-specific stimulatory effect on immunoglobulin synthesis. This would not only explain the high IgM levels seen in these infections but also the occurrence of anomalous antibodies eg autoantibodies.

The production of a B cell mitogen would be expected to lead to an increase in lymphocyte DNA synthesis, and this has been observed in patients with malaria which is a disease manifesting similar immunological symptoms. One can also postulate that this stimulation of the disordered immunoglobulin response in trypanosomiasis could benefit the parasite by swamping the specific immunological response aimed at its destruction, while it could also explain the apparent heightened resistance to concurrent infections.

### III MATERIALS AND METHODS

#### A. BUFFERS

##### 1. Borate buffer, pH 8.0

Boric acid (9.03g), sodium chloride (2.13g) and sodium borate (5.18g) were dissolved in distilled water and the volume made up to 1 litre. The pH was adjusted to pH 8.0.

##### 2. Borate buffer, pH 8.6

A stock solution (1 litre) containing 62g boric acid and 20g sodium hydroxide was prepared. A double strength borate buffer solution containing 150ml 0.1N HCl, 350ml stock borate solution and 500ml saline was diluted with an equal volume of distilled water prior to use. The pH was adjusted to pH 8.6.

##### 3. Buffered glycerol, pH 8-9

This was prepared by dissolving 0.0729g sodium bicarbonate and 0.016g sodium carbonate in 10ml distilled water. Glycerol was then added to a final volume of 100ml.

##### 4. Ca-Mg buffered diluent, pH 7.2

5,5 diethylbarbituric acid (5.75g) was dissolved in 500ml hot distilled water. To this solution 85.0g sodium chloride, 2.0g sodium, 5.5 diethylbarbiturate, 1.68g magnesium chloride and 0.28g calcium chloride were added, and the volume made up to 2 litres. This stock solution was diluted 1 in 5 with distilled water prior to use. The pH was adjusted to pH 7.2.

5. Glycine-saline buffer, pH 8.2

The pH of 975ml 0.1M glycine was adjusted to pH 8.2 with 1N NaOH. To this solution 10g sodium chloride was added and the volume adjusted to 1 litre with distilled water.

6. Phosphate buffered saline, pH 7.2

The pH of a solution of 286ml 0.15M  $\text{Na}_2\text{HPO}_4$ , 90ml 0.15M  $\text{KH}_2\text{PO}_4$  and 376ml 0.15M NaCl was adjusted to pH 7.2.

7. Phosphate buffered saline, pH 7.3

Forty ml 0.5M  $\text{Na}_2\text{HPO}_4$  and 10ml 0.5M  $\text{KH}_2\text{PO}_4$  were made up to 500ml with distilled water. To this solution 11g sodium chloride and 0.1g sodium azide were added, and the pH was adjusted to pH 7.3

8. Phosphate buffered saline, pH 7.6

Sodium chloride (8.5g), disodium hydrogen phosphate (1.28g) and sodium dihydrogen phosphate (0.156g) were dissolved in distilled water and the pH was adjusted to pH 7.6

9. Phosphate buffered saline, pH 8.0

Sodium chloride (2.55g), and sodium dihydrogen phosphate (0.468g) and disodium hydrogen phosphate (20.414g) were dissolved in distilled water, and the volume made up to 1 litre. The pH was adjusted to pH 8.0.

10 Phosphate buffered saline with glycerol and heparin, pH 8.0

To 100ml phosphate buffered saline pH 8.0, 1.0g D-glucose and 0.1ml Heparin (50001.U./ml) were added.

## B. TECHNIQUES WITH TRYPANOSOMES.

### 1. Maintenance and Purification of Trypanosomes.

A strain of T. equiperdum obtained from Dr. R.D. Bigalke of the Onderstepoort Research Institute, Pretoria, was maintained in the laboratory by blood passage in white mice, and stabulates (Herbert et al 1968) were preserved in liquid nitrogen. The severity of the trypanosome infections was tested by microscopic examination of the mouse tail blood. When the parasitaemia was considerable (3 - 4 days after infection), the mice were exsanguinated by cardiac puncture under ether anaesthesia, using 10 I.U./ml Heparin as an anticoagulant. For further passage the blood was diluted until 1 - 4 trypanosomes were observed in the microscope field (ocular lens 15x, and objective 40x). Mice were then injected intravenously with volumes varying from 0.05 to 0.2ml of dilute blood. The blood from infected mice was diluted 1:3 with cold phosphate buffered saline (PBS) pH 8.0 containing 1% glucose and 10 I.units of heparin/ml i.e. phosphate buffered saline with glucose and heparin (PBSGH). Purification of trypanosomes for the preparation of trypanosome clones was carried out following Lanham (1968).

DEAE cellulose was activated as described in the next section, and equilibrated with PBS at pH 8.0. The slurry was then washed three times with PBSGH, and poured into a glass column (4 x 10 cm), fitted with a sintered glass disc. Excess liquid was allowed to drain from the column, and the surface of the DEAE cellulose was covered by 3-4 cm layer of buffer.

The diluted blood was carefully layered on to the surface of the cellulose, and the head of buffer maintained with PBSGH. Trypanosomes were observed as a yellow band migrating in the DEAE cellulose and were collected on elution until microscopic examination proved negative.

The trypanosomes were centrifuged at 3000 rpm for 10 minutes at 4°C, and were then washed 6 times with PBSGH buffer. Cooling of the suspension to 4°C was found to be essential in order to retain the viability of the trypanosomes, since at room temperature their rate of metabolism was extremely high resulting in the utilization of all metabolites, and the accumulation of waste products. (Lumsden et al 1973).

Purified trypanosomes were cryopreserved in liquid nitrogen. Glycerol was added as a cryopreservative to the suspension to a final volume of 7.5% by slowly mixing the required glycerol volume with the trypanosome suspension. Plastic ampoules were completely filled with 0.3ml of the trypanosome suspension, labelled and quickly cooled in liquid nitrogen. The ampoules were kept in a liquid nitrogen cannister until required, when they were quickly thawed at 37°C. The trypanosomes remained viable for periods of at least nine months.



## 2. Activation of DEAE Cellulose.

DEAE Cellulose, an anionic exchange cellulose was precycled prior to use. This was in order to swell the dried cellulose in a reproducible manner, so that it could become fully accessible to charged solutes. Acidic treatment was followed by an alkaline treatment, since the former converted the cellulose to its fully charged form, whilst the latter treatment converted DEAE into the free base form. (Whatman advanced ion-exchange cellulose brochure).

Approximately 100g of dried DEAE cellulose (Merck E, Darmstadt, Germany), was stirred into 1500ml of 0.5N HCL, the suspension stirred for 2 minutes and left for 30 minutes. The supernatant was decanted and the cellulose washed with distilled water until the pH remained at 4.0. The material was then treated with 0.5M NaOH (1500ml) for 30 minutes. The cellulose was then washed, as before, until a pH of 8.0 was obtained. It was stored at 4°C until use.

Prior to use, the cellulose was equilibrated first with PBS pH 8.0 and finally with PBSGH pH 8.0.

## 3. Preparation of Trypanosome Clones.

Antigenically similar populations of trypanosomes i.e. clones, were obtained by passage initiated from a single trypanosome. (Lumsden et al 1973). A trypanosome-infected mouse with marked parasitaemia was bled by cardiac puncture, under ether anaesthesia, and the blood diluted in PBS pH 8.0 until small drops placed on a microscope slide contained a single organism. The drop was small enough to be entirely included in the microscope field, and the viewing was rapid

to prevent.../

to prevent evaporation of the drop. Confirmation of the presence of a single organism was made by another observer.

An additional droplet of diluent was placed over the trypanosome-containing drop and they were drawn up into a 1 ml syringe previously filled with 0.1 ml diluent. The recipient mouse was inoculated immediately with the trypanosome suspension. Following infection of this mouse a single passage was carried out in order to prepare sufficient trypanosomes for experimental purposes.

#### 4. Routine infection of rabbits with *T. equiperdum*.

Adult white rabbits (progeny of a cross between New Zealand and commercial hybrid strains) were infected intramuscularly with between  $10^6$  -  $10^7$  trypanosomes/ml. The rabbits were bled from the marginal vein of the ear, prior to infection and at intervals over the infection period. Sera were stored at  $-18^{\circ}\text{C}$ .

#### 5. Inactivation of trypanosomes and immunization of rabbits.

Trypanosomes were inactivated following the method of Fernandes et al (1965). Purified trypanosomes in PBSGH (0.1 ml of  $5 \times 10^8$  trypanosomes/ml were inactivated with  $200 \mu\text{g}$  Actinomycin D (P-L Biochemicals, Inc, Milwaukee, Wisconsin) for 45 minutes at  $37^{\circ}\text{C}$ . The Actinomycin D was dissolved in 0.8 ml M199 medium (Difco Lab., Detroit, Michigan). The parasites were then washed twice in PBSGH and resuspended at concentrations between  $5 \times 10^6$  -  $1 \times 10^9$  trypanosomes per ml.

Rabbits were infected intramuscularly with the inactivated trypanosomes at concentrations of  $5 \times 10^6$ ,  $1 \times 10^7$  and  $1 \times 10^8$  trypanosomes per rabbit, once a week for three weeks. Inactivated trypanosomes were freshly prepared for each immunization injection.

After three immunization injections rabbits were challenged with  $10^3$  -  $10^4$  viable trypanosomes. Positive controls were carried out by injecting unimmunized animals with an identical concentration of viable trypanosomes. Rabbits were bled at weekly intervals.

#### C. PURIFICATION OF RABBIT IgM, IgG AND THE PREPARATION OF ANTISERA.

##### 1. Preparation of a Sephadex G200 column.

###### Preparation of the Gel

Sephadex gel G200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was swollen in excess distilled water with intermittent stirring and decantation, for 3 days at room temperature. 1 gm of dried gel produced a total volume of about 30 ml of swollen gel. After swelling the gel was washed with PBS pH 7.3, 0.05M, containing 0.02% sodium azide.

###### Packing the G200 Column

After the 100 x 4.5 cm column had been mounted vertically the outlet was closed with a narrow piece of tubing approximately the length of the column. The degassed slurry, dilute enough to allow air bubbles to rise through the gel, was poured slowly into the column.

The outlet tubing was then positioned 10 cms below the liquid level in the column (fig.2), and opened to allow the excess buffer to flow out slowly. The remaining slurry was poured into the column as the excess buffer drained out.

When the column had been satisfactorily packed, the outlet was closed and the top flow adaptor fitted into place ensuring that no air bubbles were trapped under the nylon net. The column was then equilibrated with phosphate buffer, 0.05M, pH 7.3, for 24 hours prior to use.

## 2. Preparation of a Zone Electrophoresis Column

### Preparation of the Density Gradient.

In most experiments borate buffer pH 8.6 was used. The apparatus was assembled as shown in fig. 3. The buffer was slowly introduced into the apparatus through inlet A, until the level reached the side arms of the electrode tubes, ensuring that no air bubbles were formed. A solution of 40% sucrose in borate buffer was then added in a similar way until the level in the R.H.S. was at C. Buffer was then added to the L.H.S. limb, to just below the stopcock on the RHS, and about 2 mm above the capillary. (F) The stopcock was closed. Sucrose (40%) was then run out through H to gain a sharp boundary at the top of the capillary.

Mixing flasks were assembled such that the higher flask (1) contained 170 ml of 40% sucrose, and the lower flask (2) contained 160 ml of buffer. Air bubbles were removed from the tubing leading to the column, and the tubing was connected to H.

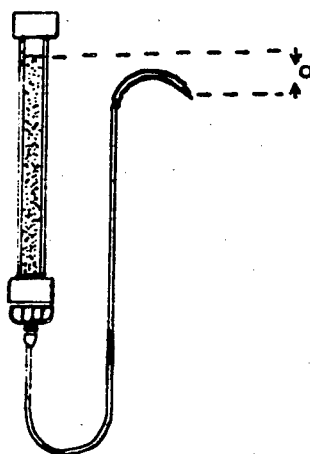


Fig. 2. The packing of the Sephadex G200 column. a, operating pressure.

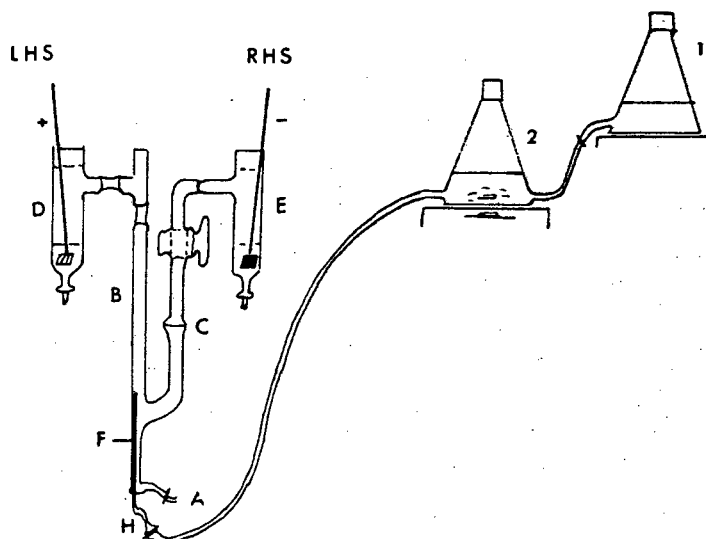


Fig. 3. Zone electrophoresis apparatus. A, side arm; B, electrophoresis column; C, level of 40% sucrose during the formation of the density gradient; D & E, electrode vessels containing the silver - silver chloride electrodes; F, capillary tube; H, outlet; 1, flask containing 40% sucrose; 2, flask containing buffer.

Both the tap between flasks (1) and (2), and the tap at H were opened to allow the sucrose to flow into the buffer solution, and the solution to enter the column. The gradient was allowed to run until only about 20 ml of sucrose was left in flask (1). Approximately 5 ml of the lower part of the gradient was then run out of the column through H. This ensured that the sucrose concentration at the bottom of the column was about 35%.

#### Introduction of the Sample.

Two bijou bottles containing i) 37% sucrose and ii) 40% sucrose were prepared. Sugar and phenol red were added to the sample (maximum concentration of 10 mg and maximum volume of 2 ml) until a drop of sample sank when added to the 37% sucrose, and floated when added to 40% sucrose. The sample was then introduced very slowly through H using a syringe. The outlet H was then firmly closed.

Reversible silver-silver chloride electrodes were placed in the electrode vessels, and covered with a saturated solution of sodium chloride. The main stopcock was then gently opened, and buffer was removed or added to the appropriate electrode vessel until the sample was about 2 inches above the capillary tube. The apparatus was allowed to stand 2 - 3 hours to establish a density gradient through the sample layer, before the current was switched on.

A voltage gradient of 3 - 4 V/cm was applied with the positive electrode being on the left. A fan was installed to reduce the heat generated by the current.

### Preparation of silver-silver chloride Electrodes.

Silver-silver chloride electrodes were constructed from strips of silver wire mesh, which were coated with a layer of silver chloride, by immersing them in 0.1 N HCl saturated with NaCl and connecting them to the positive pole of a source of direct current.

The electrode with the heaviest silver chloride deposit was connected to the negative pole. Electrodes were alternated every 24 hours by gently closing the stopcock and interchanging them.

### 3. Purification of Rabbit IgM

The purification of rabbit IgM was accomplished by a combination of euglobulin precipitation, gel filtration and zone electrophoresis techniques.

#### Euglobulin Preparation and Gel Filtration.

3 (a) The serum (10 ml) from rabbits infected with T. equiperdum was first centrifuged at 25000 g for 1 hour at 0°C, in order to remove the lipoproteins (Campbell et al 1970), and was then twice precipitated with an equal volume of 4M ammonium sulphate at pH 7.4 (Nowotny 1969). An average yield of 20 mg protein/ml of serum was obtained after ammonium sulphate precipitation.

Ascending gel filtration was then carried out by pumping a maximum of 200 mg of the redissolved protein precipitate (2-3 ml) on a 4.5 x 100 cm column packed with Sephadex G200. A phosphate buffer 0.05M, pH 7.3 was used for elution at a flow rate of 24 ml/hr. Absorbance at 280 nm was monitored by a LKB Uvicord II, (LKB, Bromma, Sweden) and 5 ml fractions were collected.

IgM-containing fractions were pooled, concentrated from 80-100 ml to 5-10 ml by ultrafiltration, using an Amicon XM 100A membrane in a Model 52 Stirred cell, at 15psi and 4°C. (Amicon Corporation, Lexington, Massachusetts.)

An average macroglobulin concentration of 2 - 4 mg/ml of serum was obtained.

### 3 (b) Zone Electrophoresis

The final IgM purification step was carried out by Zone electrophoresis (Polson and Russell 1967 and van Regenmortel 1972) at either pH 6.1 or at pH 8.6.

An aliquot of the concentrated IgM fraction (maximum volume of 2 ml) was introduced into a 0 -40% sucrose gradient prepared as outlined previously. Electrophoresis proceeded for 3 days at a potential of 200V, with alternation of the silver chloride electrodes every 24 hours. Fractions (2ml) were collected manually and the absorption measured at 280nm on a Pye Unicam Sp 1700 U.V. spectrophotometer.

(Pye Unicam Ltd., Cambridge, England). IgM containing fractions were then dialysed against the respective buffer and concentrated by ultrafiltration to 1 - 2 ml. The purity of the IgM was tested by immunoelectrophoresis.

### 4. Reduction of 19S IgM

#### a) Cysteine Reduction

IgM (5mg/ml) was reduced at room temperature for 8 minutes with 0.05M cysteine in Tris buffer pH 8.6, 0.1M (Miller and Metzger 1965). The reaction mixture was alkylated with a 10% excess of iodoacetamide and was applied to a Sephadex G200 column.



b) Mercaptoethanol Reduction.

Rabbit IgM (2mg/ml) was reduced with an equal volume of 0.5M mercaptoethanol for 1 hour at 37°C. (Banatvala et al 1967).

c) Dithioerythritol Reduction

19S IgM (0.9 of 3 mg/ml) was reduced for 1 hour at room temperature with 2mM DTT (0.1ml of 20mM DTT) in 0.2M Tris buffer pH 8.0 (Parkhouse 1975). The reductant was cooled to 0°C and then alkylated by adding a 50% molar excess of iodoacetamide. (0.11ml of 30mM iodoacetamide).

d) Purification of 7S and 13.4S IgM.

IgM samples (0.15ml) reduced with 2mM DTT were centrifuged on a sucrose gradient at 26000 rpm for 24 hours at 4°C. The 7S IgM fractions were pooled, dialysed against normal saline overnight, and concentrated by ultrafiltration, using an Amicon XM 50A, 25 mm membrane. 13.4S samples were treated in a similar way. Preparations were analysed by immunoelectrophoresis.

5. Sucrose Density Gradient Centrifugation.

Separations of 7S and 19S components was performed on 0-40% sucrose gradients prepared in 19ml Beckman cellulose nitrate centrifuge tubes, using an LKB gradient former. Sample volumes of 0.1 to 0.2 ml were placed on the gradients, and the gradients were then centrifuged at 26000 rpm for 24 hours at 4°C in a Beckman SW 27 rotor in a Spinco model L3 -50 Ultracentrifuge.

Fractionation of the gradients was carried out by pumping 60% sucrose into the bottom of the centrifuge tube.

Fractions of 1 ml were collected and monitored at 280nm using the Isco density gradient fractionator Model 640. (Isco, Lincoln, Nebraska). The refractive indices of the sucrose fractions were determined using an Atago ABBE refractometer (Atago, Japan).

Pooled fractions corresponding to the 7S and 19S components were dialysed overnight against phosphate buffered saline, 0.05M, pH 7.3 or against normal saline, and were then concentrated by ultrafiltration using Amicon XM 100 A 25 mm membrane, at 15psi at 4°C.

## 6. Preparation of Goat Antisera

### a) Preparation of Goat Anti Rabbit IgM Serum.

Anti rabbit IgM was prepared by immunizing a goat with purified rabbit IgM emulsified in Freund's incomplete adjuvant. The goat received a total of 8mg of IgM over a period of 3 weeks, and was bled from the jugular vein at fortnightly intervals after the last injection.

### b) Absorption of Goat Anti Rabbit IgM Serum.

#### i) Neonatal Serum Absorption

One volume of goat anti-rabbit IgM was absorbed with a  $\frac{1}{2}$  volume of neonatal rabbit serum collected from 3, one day old rabbits, for 1 hour at 37°C. The precipitate was removed by centrifugation and the absorbed goat antiserum tested by immuno-electrophoresis.

ii) Dialysed NRS.Absorption.

Goat anti rabbit IgM serum was absorbed for 1 hour at room temperature with an equal volume of NRS which had been dialysed for 3 days against distilled water at 4°C. The precipitate was removed by centrifugation, and the antiserum analysed by immunoelectrophoresis.

iii) Absorption of Goat antiserum with NRS Precipitate.

Goat antiserum was absorbed for one hour at room temperature with the precipitate formed after dialysis of 10 ml of NRS against 0.105M sodium chloride and 0.00012M acetate buffer at pH 5.4 (Brown et al, 1954).

iv) IgG Absorptions

Goat antiserum (10 ml) was absorbed for one hour at room temperature with rabbit IgG prepared by the rivanol method, at a concentration of 1.5 mg IgG/ml antiserum.

c) Preparation of Goat Anti NRS Serum.

Anti rabbit serum was prepared by immunizing a goat with a total of 4 ml NRS emulsified in Freund's incomplete adjuvant over a period of 8 weeks, (4 injections). The goat was bled at fortnightly intervals after the last injection. Booster injections were given every 2 - 3 months.

d) Preparation of Goat Anti Rabbit IgG Serum.

A goat was immunized with a total of 14 mg of rabbit IgG, prepared by the rivanol technique, emulsified in Freund's incomplete adjuvant over a period of 9 weeks (4 injections). Antiserum was collected at fortnightly intervals, and booster injections of 2 - 4 mg IgG were given when necessary. The goat antiserum was absorbed with

purified.... /

purified IgM at a conc of 1.25 mg IgM / ml antiserum for one hour at room temperature.

#### 7. Preparation of Rabbit IgG

Rabbit IgG was isolated from rabbit serum using Rivanol (2-ethoxy-6:9-diaminoacridine lactate) by a procedure developed from the original work of Horesji & Smetana (1956) and Stastny and Horesji (1961).

The pH of rabbit serum was adjusted to between pH 8.0 and pH 9.0. In this pH range Rivanol reacts with serum proteins having a pI of less than 5.5, eg albumin,  $\beta$  lipoprotein and  $\alpha$ , glycoprotein etc. IgG with a pI of 7.3 has a low negative charge, which results in insufficient electrostatic interaction, between the dye molecules and the IgG molecules, to cause protein aggregation.

Three and a half volumes of 0.4% Rivanol was then added dropwise to the alkaline serum (10 ml) and the precipitate so formed removed by centrifugation. The precipitate was suspended in 3.5 volumes of distilled water, and stirred to extract further IgG, then centrifuged. The two supernatants were pooled, and the Rivanol was removed by precipitation with 1.5 volumes of saturated Potassium bromide solution (Schultze & Heremans 1966).

The IgG was precipitated from this supernatant with 1.6M ammonium sulphate solution. The precipitate, so formed, was resuspended and reprecipitated twice more. The resulting precipitate contained a high concentration of IgG, which was then dialyzed, lyophilized and stored at 0°C.

## 8. Immunoelectrophoresis of Serum Proteins.

Micro immunoelectrophoresis was carried out based on the methods of Scheidegger (1955) and the Gelman handbook (1970).

Glass slides were cleared with methanol and placed in Gelman immuno frames (Gelman Instrument Co., Ann Arbor, Michigan, U.S.A.) on a levelling stand. Each slide was then "painted" with an adhesive agar made up of 0.1% agar, 0.05% glycerol and 0.01% sodium azide in distilled water. After allowing the agar glue to dry, 10ml of hot 1.0% agarose in 0.025M Tris buffer pH 8.1, containing 0.01% sodium azide was spread over each row of 3 glass slides. After the gel had set, an immunoelectrophoretic pattern was then punched onto the hardened gel, using the Gelman gel punch. The pattern consisted of 3 wells 1.5 mm in diameter, separated by two troughs 1.0 mm wide, with a diffusion distance, between each well and trough, of 3.6mm. The agarose from each well was removed by suction.

Antigen samples were placed in the wells using finely drawn-out Pasteur pipettes. Each frame was then placed in the electrophoresis chamber such that the wells were nearest the cathode end. Cellulose acetate wicks formed bridges between the agarose and the buffer in the chamber. Electrophoresis was carried out in 0.1M Tris buffer pH 8.1 at 5v/cm for 1.5 hours at room temperature.

At the end of the run the gel from the troughs was removed with a gel knife and the troughs were filled with antiserum. Frames were placed in a humid chamber overnight to allow the precipitin bands to develop.

Slides were then washed for 24 hours in 0.2% sodium chloride solution followed by a final rinse in distilled water to remove unreacted proteins. Moistened absorbent paper strips were then placed over the slides and the slides allowed to dry at room temperature. Staining was carried out in 0.25% Coomassie blue, and destaining in methanol: distilled water: acetic acid (45:45:10) solution.

Quantitation of serum IgM by immunoelectrophoresis was performed by electrophoresing serial dilutions of the rabbit sera against goat anti rabbit IgM serum. The last dilution at which the IgM precipitin arc was visible was used as the endpoint (Crowle 1973).

Serial dilutions of IgM standards were used to calibrate the technique.

#### D QUANTITATION OF RABBIT IgM AND IgG.

##### 1. Determination of IgM Concentrations by Quantitative gel Precipitin Tests.

IgM concentrations were determined by the quantitative gel diffusion test of Polson (1958), Van Regenmortel (1966.)

A perspex apparatus with a series of ten circular holes having the following dimensions was used; 0.5 cm in diameter x 1 cm in length (Fig.4). Prior to the assembly of the apparatus the surfaces of the five sections were well greased, care being taken to ensure that the grease did not enter the holes. After assembly the holes were meticulously cleaned. The centre wells were filled with 0.2 ml of 0.75% agarose in barbital buffer 0.05M, pH 8.6.

by moving.../

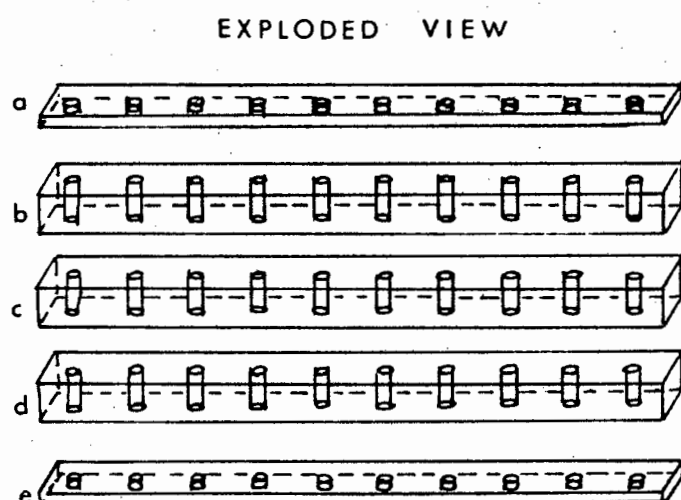


Fig. 4. Apparatus for quantitative gel diffusion. Sections (a) and (e), form the top and bottom lids respectively. The holes in (b), serve as antigen reservoirs, and those in (d), as antiserum reservoirs. The holes in section (c), serve as the agarose containers.

by moving the lower section (d) off centre.

The centre wells (c) were then  $\frac{3}{4}$  closed by the slow off-centre movement of layer (b), and the apparatus placed at 4°C for 10 - 15 minutes, to allow the agarose to set completely. The centre wells were then completely closed, and excess agarose removed from (b) layer by suction.

Suitable antigen dilutions (IgM or IgG standards or experimental sera) were placed in the wells of layer (b), this being closed by moving section (a) off centre. In a similar way the diluted antiserum was placed in the wells of layer (d).

The three centre sections (b,c,d) were carefully aligned and diffusion allowed to occur at room temperature for 48 hours. The positions of the precipitin bands relative to the menisci were measured with a Nikon microcomparator. Band position is a linear function of the logarithm of the ratio of antigen to antibody concentration. Calibration curves were obtained by plotting the positions of precipitin bands formed by serial twofold dilutions of purified IgM or IgG against the corresponding constant antibody.



## 2. Laurell Electrophoresis

Laurell electrophoresis was carried out by a method based on that of Weeke (1973b).

Molten 1 % agarose in 0.01M barbital-glycin-Tris buffer, pH 8.8 (5.6ml) was pipetted onto each precoated glass plate (50x75mm) on a levelling stand. The plates were precoated with molten agarose with the aid of a small paint brush. The agarose was allowed to set and formed a layer 1.5mm in depth. Two wells 0.5mm in diameter were punched into the agarose and filled with 1.5  $\mu$ l of either IgM standard preparations or dilute rabbit serum, by means of a Hamilton syringe. The slides were electrophoresed in an Laurell electrophoresis chamber, 28.0cm long x 9.0cm deep x 13.5cm wide, with movable supports 2 cm apart. Lint wicks formed the bridges between the 0.02M barbital-glycine-Tris buffer and the agarose. Electrophoresis was carried out for 50 minutes at 6V/cm.

A 10 mm strip of agarose was then removed from each side of the plate, and the remaining agarose containing the two wells divided into 2. Each slab, 50x19mm, was then placed onto a second precoated glass plate, and 4.2ml of 1% agarose containing the antiserum was pipetted onto the remaining part of the plate. The plates were electrophoresed at 1V/cm overnight (16 hours), in a direction at right angles to the first dimension.

The following morning the plates were pressed, washed, pressed, dried, stained, and destained as follows:

The wells on the plates were filled with distilled water, and moistened blotting paper strips placed over the plates such that all air bubbles were excluded. The plates were then placed under several layers of dry blotting paper, and pressed under a book for 20 minutes. After pressing the plates were twice washed in 0.1M sodium chloride for 20 minutes with constant stirring and then once in distilled water for 20 minutes, and finally repressed and dried in front of a hot air fan.

The precipitin peaks were stained in 0.5% Coomassie brilliant blue R250 for 20 minutes, and destained in a solution of 45 parts of 96% ethanol: 10 parts of glacial acetic acid: 45 parts of distilled water.

Both the heights of the peaks, and the areas under the peaks were measured.

Variations of the technique outlined, included the use of longer staining times of 40 and 60 minutes, Ponceau S, Amido black, Procion blue, and Coomassie R stains, precipitin brighteners eg 0.5% phosphotungstic acid at pH2, and pH 5, 0.5% phosphomolybdic acid at pH2 and pH5 and finally formalinization of the rabbit sera with 0.01% to 0.2% formaldehyde.

### 3. Rocket Electrophoresis

The technique used for rocket electrophoresis, was based on that used in Laurell electrophoresis (Weeke, 1973a). Molten 1% agarose in 0.01M barbital-glycine - Tris buffer, pH 8.8 containing anti rabbit IgM serum at a final dilution of  $1/256$ , (5.6ml), was pipetted onto precoated glass plates (50 x 75mm). Six wells, 2 mm in diameter, were punched 5mm apart on the plate, and filled with 2  $\mu$ l of the IgM standards or dilute rabbit sera. The plates were placed in a Gelman deluxe electrophoresis chamber (Gelman Instrument Company, Ann Arbor, Michigan, U.S.A.), filled with 1 litre of 0.02M barbital-glycine-Tris buffer pH8.8 and with absorbent lint wicks connecting the buffer and the agarose. Electrophoresis was carried out for 16 hours at 2V/cm.

Gels were then pressed, washed, pressed, dried, stained and destained as outlined for Laurell electrophoresis. The heights of the precipitin peaks were recorded in mm.

### 4. Analytical Ultracentrifugation.

#### a) Calibration of the Area under a Schlieren Peak by Fringe Shifts.

A 4mg/ml bovine serum albumin (BSA) standard was dialysed for 48 hours against normal saline. The absolute concentration of this standard was measured by interference optics in a double sectored synthetic boundary cell, of which one sector of the centre-piece was filled with 0.15ml of BSA solution, and the other sector with 0.45 ml of the buffer. The sample was centrifuged at 10000 rpm in a Beckman

Model E.... /

Model E analytical ultracentrifuge. The total fringe shift, comprised of the whole fringe count and the fractional fringe was determined using a Nikon micro-comparator. The protein concentration was calculated on the basis of:

Shift of one fringe = 0.25mg protein/ml.

A Schlieren pattern of the BSA standard at a schlieren angle of  $60^\circ$  was also obtained using the double sector cell, and the area under the schlieren peak measured by tracing the enlarged image onto graph paper. Corrections were made for radial dilution and magnification. (Table 1.)

b) Determination of 19S Concentrations in Rabbit Sera.

Rabbit sera were diluted 1 in 5 in saline and centrifuged at 56,000 rpm in an analytical Rotor D. Two cells were used simultaneously, one with quartz plain windows, and the other with a quartz  $1^\circ$  wedge window. Schlieren patterns at an angle of  $60^\circ$  were photographed every four minutes after reaching speed. The areas under the schlieren peaks were measured and corrected as above, and compared with the BSA standard.

c) Determination of Sedimentation Coefficients.

Samples were centrifuged and schlieren patterns photographed as outlined above. Using the Nikon comparator the radial distance of the apex of each schlieren peak from the centre of rotation was measured for each photograph (Table 2 A and B).

TABLE I.

Calibration of the area under a schlieren peak by  
fringe shifts.

Whole fringe count	Fractional fringe	Corrected fringe	Conc. (Mg/ml)	Area Sq.inches
14	$\frac{\gamma_f}{\gamma_v} = \frac{0.135}{0.27}$	15.95	3.98	32.12
	= 0.5			

TABLE 2

## Determination of Sedimentation Coefficients.

Table 2A

Photo No.	Time (min )	Position of boundary (mm)	Position of outer reference (mm)	Distance of boundary from outer reference (mm)
2	8	17.410	1.190	16.200
3	12	14.732	0.528	14.205
4	16	12.690	0.537	12.153
5	20	13.851	4.933	8.918

Table 2B

Photo No.	Distance of boundary from outer reference (mm)	Distance $\times$ M. factor	Distance in cm	Distance of boundary from centre of rotation ( $\alpha$ )	Log $\alpha$ 10
2	16.200	7.579	0.758	6.542	0.8157
3	14.204	6.637	0.664	6.636	0.8219
4	12.153	5.679	0.568	6.732	0.8219
5	8.918	4.169	0.417	6.883	0.8378

The slope of a plot of  $\text{Log}_{10}$  distance against time in minutes was determined, and the  $S(\text{obs})$  calculated from the following equation:

$$S_{\text{obs}} = \frac{1}{\omega^2 r} \left( \frac{dr}{dt} \right) = \frac{2.303}{60\omega^2} \left( \frac{d \log x}{dt^1} \right)$$

Where  $t$  = time in seconds

and  $t^1$  = time in minutes.

The  $S(\text{obs})$  of IgM was found to be  $17.85 \times 10^{-13}$

It was not possible to correct the  $S(\text{obs})$  to standard conditions as accurate temperature measurements, were unfortunately not made, because of a faulty rotor temperature indicator system. Centrifugation runs were carried out in an air-conditioned room at  $19^\circ\text{C}$ , the temperature of the rotor agreeing to within  $0.5^\circ\text{C}$ . For this reason all  $S$  values included in this work are uncorrected values.

#### E. METHODS FOR THE DETECTION OF RHEUMATOID FACTORS.

##### 1. Latex Fixation.

Latex fixation was carried out both as a semi-quantitative tube test, and as a slide test, the latter being the most satisfactory.

##### a) The Latex Tube Test.

The tube test was carried out following Singer & Plotz (1956). Difco latex particles (Difco Labs, Detroit, Michigan, USA) were diluted to 1 in 10 in glycine saline buffer pH 8.2, and to which was added rabbit IgG at final concentrations of 5mg/ml and 1mg/ml. Two-fold dilutions of rabbit sera..../

rabbit sera were made in glycine saline buffer in test tubes, and an equal volume (0.2ml) of latex-IgG was added. The tubes were incubated at 56°C for 3 minutes, and the resulting agglutination was read with the naked eye.

Samples were also kept at 4°C overnight and recentrifuged and read the following morning. (Singer et al 1960). Difficulties were experienced with weak positives and non-specific agglutination in spite of attempts to stabilize the reaction. Such attempts included the addition of 0.05mg BSA/ml, and the resuspension of washed latex particles in 0.2M Tris-HCL pH 7.2 containing 0.02% PVP (Abu-Salih et al 1968).

b) The Latex Slide Test.

The latex slide test, found to be the most satisfactory with respect to sensitivity of the reaction and reduced sample volumes, was carried out following Cheng & Persellin (1973). Difco latex was diluted 1 : 25 in distilled water and was coated with rabbit IgG

Human Cohn Fr II (Miles Labs., C.T.) as outlined in table B. Rabbit sera were heated for 40 min at 56°C, and diluted in 0.1M glycine saline buffer, pH 8.2.

A glass plate 20 x 20 cm was prepared as a multi-spot slide with wells 1.5 cm in diameter approximately 2 cm apart (Kidby 1974). The wells were formed by rinsing glycerol droplets off the plate which had been sprayed with a teflon spray (PTFE spray, Pampus, Fluoroplast Ltd, Stoke-on-Trent, England).



A small volume of the formalinized SRBC's was washed and checked for adequate formalinization indicated by a reddish-brown colour. After suitable formalinization had been achieved the SRBC's were washed 6 times in normal saline and packed, the packed cells being finally resuspended at 25% in normal saline. The cells were kept at 4°C, with 0.1% sodium azide as a preservative.

#### Sensitization of the Formalinized SRBC's.

Formalinized SRBCs were tanned by diluting the cells to 2.5% and adding an equal volume of 1 in 20,000 tannic acid (0.05 mg/ml) (Daniel & Stavitsky 1964, Weir 1973). The cells were incubated at 37°C for 15 minutes, and were then washed twice in PBS pH 7.2, 0.15M. Sensitization of the tanned cells was performed by incubating the cells for 30 minutes at 37°C, in the presence of IgG preparations, diluted in PBS pH 7.2, as outlined in Table 8. The control cells although, not sensitized with IgG, were treated in a similar way.

After sensitization the cells were again washed twice in either 1 in 100, or 1 in 200 inactivated and absorbed normal rabbit serum in PBS, and were finally resuspended at 2.5%. The sensitized cell preparations were found to be stable at 4°C for two days.

#### Preparation of normal rabbit serum (NRS) and Test Sera.

Both NRS and test sera were heat inactivated by heating to 56°C for 40 minutes, and were absorbed with the tanned uncoated SRBC's (Control cells) in order to remove heterophile agglutinins. An equal volume of the control cells was added to the sera, at room temperature, and after 10 minutes the cells were removed by centrifugation.

### Titration of the Test Sera.

Twofold dilutions ( $50\ \mu\text{l}$ ) of the sera were made in the NRS-PBS diluent in U shaped perspex microtitre plates (Cooke Instruments, Zurich, Switzerland). One volume ( $10\ \mu\text{l}$ ) of the coated cells was then added to each test well, the plate gently agitated, covered with paraffilm and left at room temperature overnight. Serum controls consisting of one volume of the rabbit sera at the initial dilution and one volume of tanned uncoated cells, and cell controls were also prepared. Cell controls consisted of coated and uncoated cells diluted in the diluent.

Agglutination indicated by a complete carpet of cells covering the bottom of the wells, was graded as outlined in Daniel & Stavitsky (1964). Non-agglutinated cells formed a compact button at the bottom of the well. The last 2+ well in the series was found to provide the most reliable endpoint.

## F. METHODS FOR THE DETECTION OF ANTI-TRYPANOSOME ANTIBODIES.

### 1. Indirect Immunofluorescence.

Antibodies directed against the exoantigens of trypanosomes were detected by immunofluorescence (Williams et al 1963, Kimber 1966). Viable trypanosomes at a predetermined optimal concentration of  $3 \times 10^4/\text{ml}$  ( $20\ \mu\text{l}$ ) were placed in each well of teflon coated multispot glass slides (Goldman 1968, Kidby 1974). Stabilates of the organisms kept in liquid nitrogen were used throughout the test.

The trypanosome droplets were air-dried and then fixed with absolute methanol for 10 minutes. Both acetone and 5% formalin were found to be unsuitable as fixative reagents. The slides were then washed in PBS pH 7.6 for 5 minutes.

To each test well one drop of twofold diluted rabbit serum was added, and to each control well one drop of twofold diluted control serum was added with standardized glass droppers. The slides were then placed in a humid chamber, and left at room temperature for 20 minutes. After incubation the antiserum was rinsed off and the slides washed for 30 minutes with PBS pH 7.6, in a slide staining dish over a magnetic stirrer.

After allowing the slides to air-dry one drop of either dilute fluorescein conjugated sheep anti-rabbit immunoglobulin serum (Wellcome Research Labs, Beckenham, England) or dilute fluorescein conjugated goat anti rabbit IgM anti serum (Cappel Labs. Inc., <sup>n</sup>D<sub>w</sub>ington, P.A. U.S.A.) was added to each test sera. The dilutions of the fluorescein conjugated antisera were pre-determined as outlined below.

Reaction of the antigen-antibody complexes with the fluorescein reagent proceeded for 20 minutes in a humid chamber at room temperature. The slides were then rinsed and washed as before for 2 hours with several changes of buffer. After air-drying the slides were mounted in buffered glycerol, and viewed under a Zeiss fluorescent photomicroscope.

Illumination was provided by a CSI 250 W metal-halide lamp (Philips, Holland), with a spectral emission as in Fig 5. A BG 12 (Schott & Gen, Mainz, W. Germany), primary filter and two OG (Schott & Gen, Mainz, W. Germany) secondary (barrier) filters excluding UV-blue light were used. (See Fig. 6).

#### Determination of the Working Dilution of Fluorescent Reagents.

Slides were prepared and treated as outlined above, using a single dilution of test and control sera, and twofold dilutions of the fluorescent reagent. The working dilution of the fluorescent reagent was the highest dilution which gave intense specific staining with negligible background staining.

#### 2. Direct Immunofluorescence with Rabbit IgM

Difco latex particles diluted 1 in 2.5 in glycine-saline buffer pH 8.2 were coated with rabbit IgM at a final concentration of 1mg/ml. A drop of the coated latex was air dried on a glass slide, fixed with methanol for 10 minutes and was treated with fluorescein conjugated sheep anti rabbit immunoglobulin serum. After 20 minutes the slide was washed for 1 hour with PBS pH 7.6, and viewed under the fluorescent microscope.

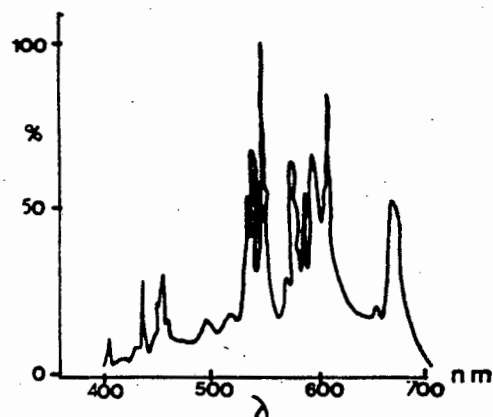


Fig. 5. Spectral emission of the CSI 250 W lamp.

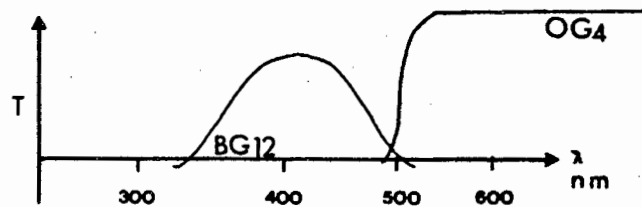


Fig. 6. Spectral transmission of the primary (BG12) and secondary (OG 4) filters.

### 3 Direct Agglutination.

Direct agglutination was performed by a modification of the method of Cunningham & Vickerman (1962).

To each of 48 wells on a teflon coated glass plate prepared as outlined previously, 20  $\mu$ l of diluted serum was added. An equal volume of purified trypanosomes ( $2.5 \times 10^8$  trypanosomes/ml) or infected blood containing  $10^7$  trypanosomes/ml was then added to each well, and the plate was incubated in a humid chamber for 50 minutes at room temperature. The agglutination was observed under a dissecting microscope (x40), and graded according to Cunningham & Vickerman (1962). Agglutination of "1" was used as the end point.

## 6. METHODS FOR THE DETECTION OF AUTOIMMUNE ANTIBODIES

### 1. Micro Complement Fixation.

Micro complement fixation was carried out by the method of Bradstreet & Taylor (1962).

#### Preparation of Reagents.

#### Titration of Haemolytic Serum and Complement

The preparation of complement and haemolytic serum must be titrated prior to use. The optimal sensitizing concentration of haemolytic serum may be defined as that dilution which gives most lysis with the highest dilution of complement. The complement titre is that dilution of complement which contains 3 units of complement i.e.  $3HC_{50}$ .

One unit of complement is contained in the dilution which gives 50% lysis at the optimal sensitizing concentration of the haemolytic serum.

In order to determine the optimal concentration of haemolytic serum and the titre of the complement, a "chessboard" titration was prepared in a perspex microtitre plate (Cooke Instruments Ltd., Zurich). Complement dilutions with a 20% difference in concentration between them from  $1/30$  to  $1/179$  and twofold dilutions of haemolytic serum from  $1/25$  to  $1/800$  were prepared. Two volumes of diluent and one volume ( $50 \mu\text{l}$ ) of complement were then added to each well in the microtitre plate such that there were six identical rows of complement dilutions on the plate. To a seventh row, three volumes of diluent were added and the plate was then left overnight at  $4^{\circ}\text{C}$ .

To 1 ml of 4% SRBC suspension was then added an equal volume of each haemolytic serum dilution. The SRBCs were then sensitized by incubating them at  $37^{\circ}\text{C}$  for ten minutes, or at room temperature for 30 minutes, and were then left overnight at  $4^{\circ}\text{C}$ .

The following morning the plate and the SRBC suspensions were incubated for 30 minutes at  $37^{\circ}\text{C}$ , and  $50 \mu\text{l}$  of each suspension was added to the appropriate row of wells in the plate. The plate was then incubated for 30 minutes at  $37^{\circ}\text{C}$  with intermittent agitation, and then placed at  $4^{\circ}\text{C}$ . After approximately 5 hours the % lysis was recorded.

Twofold dilutions ( $50 \mu\text{l}$ ) of a rabbit serum were made in the Ca-Mg buffer pH 7.2 in the microtitre plate. One volume of antigen ( $50 \mu\text{l}$ ) was added to each well, such that each row of diluted rabbit serum contained one of the serial dilutions of antigen. One volume of the complement was added to each well, and the plate left overnight at  $4^{\circ}\text{C}$ .

Antigen, antiserum and complement controls were prepared as follows:

Antigen Control.

One volume of each dilution of antigen was added to one volume of buffer, and to which was added one volume of complement.

Antiserum Control.

To one volume of each dilution of antiserum, one volume of buffer and one volume of complement were added.

Complement Controls.

Complement was diluted in buffer such that one well contained 3 complement units, one well one unit of complement and third well  $\frac{1}{2}$  unit of complement.



The 4% SRBC suspension was sensitized with an equal volume of the haemolytic serum dilution at 37°C for ten minutes and then left overnight at 4°C. The following morning, both the plate and the SRBC suspension were incubated at 37°C for 30 minutes. One volume of SRBCs was then added to each well in the microtitre plate, and to a sheep cell control well, and a further incubation at 37°C for 30 minutes was carried out with intermittent agitation of the plate. The plate was left overnight at 4°C.

The optimal dilution of antigen was then determined as the dilution giving the most complement fixation with the highest dilution of antiserum. The antigen, antiserum controls should be negative, and the complement controls graded as 4, 2, 1 on the following scale:

- 0 = no cells remaining
- 1 = 25% cells remaining
- 2 = 50% cells remaining
- 3 = 75% cells remaining
- 4 = 100% cells remaining.

#### Titration of Test Sera.

Test sera were diluted in the microtitre wells as before. To each well, one volume of the optimal antigen dilution and one volume of complement were added. The plate was then treated as outlined under the titration of antigen section. The antisera titres were taken as the final dilution that gave a reading of 2 with the optimal dilution of antigen.

2. Absorption of Sera with Trypanosomes for Micro Complement Fixation.

Sera from trypanosome infected rabbits were absorbed following Mackenzie & Boreham (1974), with crude trypanosome antigens. The sera were diluted 1 in 4 in Ca-Mg buffer to which one volume of trypanosome antigens was added, and were then incubated at 37°C for one hour. A second incubation of one hour at 4°C was followed by the removal of the trypanosome antigens by filtration. The absorbed sera were maintained at 0°C overnight.

3. Preparation of Trypanosome Antigens.

Trypanosomes, purified from terminally infected mice were frozen and thawed and finally homogenized with a ground glass homogenizer. (Seed and Gam 1967). The crude antigen fraction so formed was stored at -20°C until required.

4. Preparation of Tissue Antigens.

Normal and trypanosome infected rabbits were killed, and their livers, kidneys and hearts removed as rapidly as possible. The organs were placed in weighed beakers of normal saline on ice, and the net weight of the organs calculated. The tissues were homogenized in 4 ml saline/gm of tissue at 4°C using an M.S.E. Atomix blender (M.S.E., Crawley, Sussex, England) at  $\frac{1}{2}$  maximum speed for 15 minutes.

The suspension was centrifuged at 32,000 g for 15 minutes at 4°C. Supernatants were stored at -20°C until required.

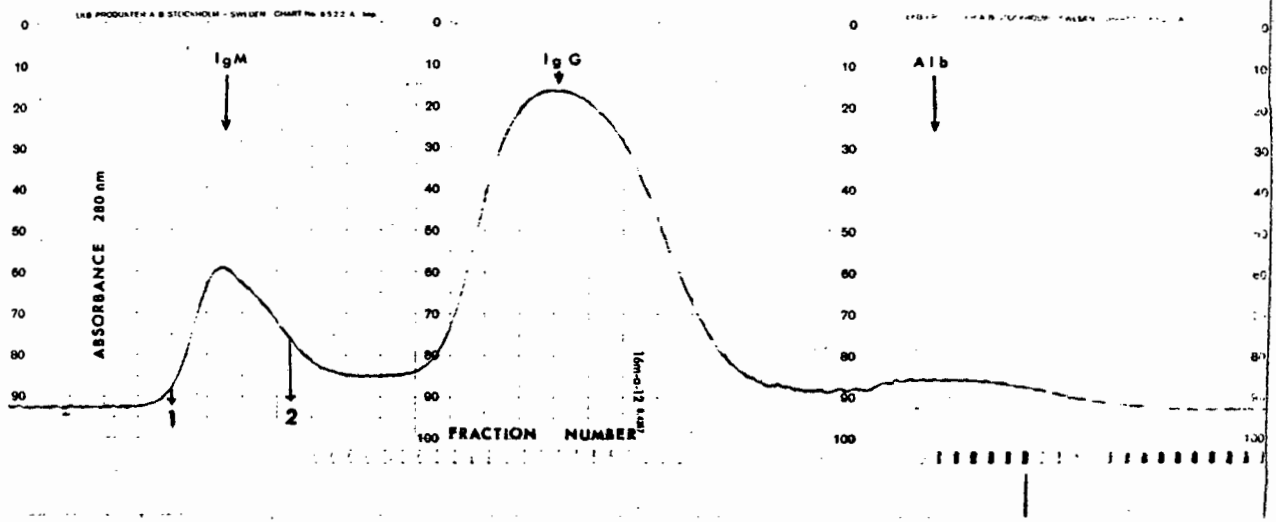


Fig. 7. Fractionation pattern of the ammonium sulphate precipitate of serum proteins (2 ml) on a Sephadex G200 column. The precipitate was derived from 10ml serum from trypanosome infected rabbits. The column was eluted with 0.05M phosphate buffer, pH 7.3, at a flow rate of 24ml/hr. Protein absorption was monitored at 280nm, and recorded at a chart speed of 10mm/hr. IgM fractions between arrows 1 and 2 were pooled (approximately 80ml) and ultrafiltrated to 5ml.

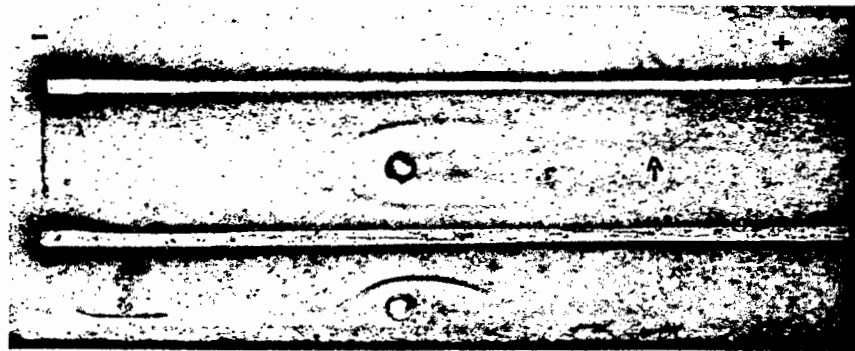


Fig. 8. Immuno-electrophoretic pattern of the IgM preparation obtained after gel filtration. Goat antiserum to rabbit serum proteins diluted  $\frac{1}{2}$  was used in the troughs. Centre well: IgM from a Sephadex G200 column; Lower well; purified IgM after gel filtration and zone electrophoresis. A faint  $\alpha_2$  macroglobulin precipitin band may be seen (see arrow).

When the partially pure IgM was tested by immunoelectrophoresis against goat antiserum to rabbit serum proteins both IgM and  $\alpha_2$  macroglobulin precipitin bands were visible. (Fig. 8).

From later work (see Table 5), it was estimated that 10ml of rabbit serum from the peak of macroglobulin-aemia contained between 50-60mg IgM. A total of 20mg of partially pure IgM was isolated after gel filtration of the ammonium sulphate precipitate derived from 10ml serum.

The final step in the purification of rabbit IgM was zone electrophoresis on a sucrose gradient. Zone electrophoresis (ZE) was initially performed at pH 6.1, a pH close to the isoelectric point of IgM, in order to reduce the migration rate of the IgM relative to that of  $\alpha_2$  macroglobulin and thereby achieve better separation. Partially pure IgM (8mg) was electrophoresed for three days in a sucrose gradient prepared with 0.03M phosphate buffer pH 6.1. Fractions (2ml) were collected manually and the absorption measured at 280nm, on a Unicam SP 1700 spectrophotometer. A single protein peak was detected, the 3 fractions of which were pooled and dialysed overnight at 4°C, against phosphate buffer pH 6.1, and finally concentrated to 1 ml by ultrafiltration. However, on immunoelectrophoretic analysis of the undiluted sample no precipitin bands were formed against goat anti-rabbit IgM serum. Since pH 6.1 is close to the isoelectric point of IgM, and.../

and in view of its lability, it is likely that the IgM was denatured.

Further attempts to purify the IgM by means of zone electrophoresis at pH 8.6 instead of pH 6.1 were conducted. Fig 9 illustrates the fractionation pattern of 10 mg IgM from a ZE column. Fractions 14-17 were pooled and dialysed overnight at 4°C against borate buffer pH 8.6. The sample was concentrated to 2ml by ultrafiltration and analysed by immunoelectrophoresis. A single precipitin line formed between the ZE sample and goat antiserum to rabbit serum proteins (Fig 10). The position of the band corresponded to that of rabbit IgM.

A total of 2.5mg purified IgM was obtained after zone electrophoresis of 10 mg partially pure IgM. Since 20mg partially pure IgM was obtained after gel filtration of 10ml rabbit serum, a total of 5mg of pure IgM was therefore prepared from 10ml serum. The probable <sup>IgM</sup> yield was therefore approximately 10%.

The sedimentation coefficient of the IgM was determined as 17.9S by analytical ultracentrifugation.

Anti rabbit IgM antiserum was prepared by injecting this material into a goat. When the resulting goat antiserum was tested by immunoelectrophoresis against normal rabbit serum (Fig 21) it appeared that the IgM used for immunization was still contaminated with traces of  $\alpha_2$  macroglobulin and  $\beta$  lipoprotein. For this reason attempts were made to improve the purity by

increasing the .... /

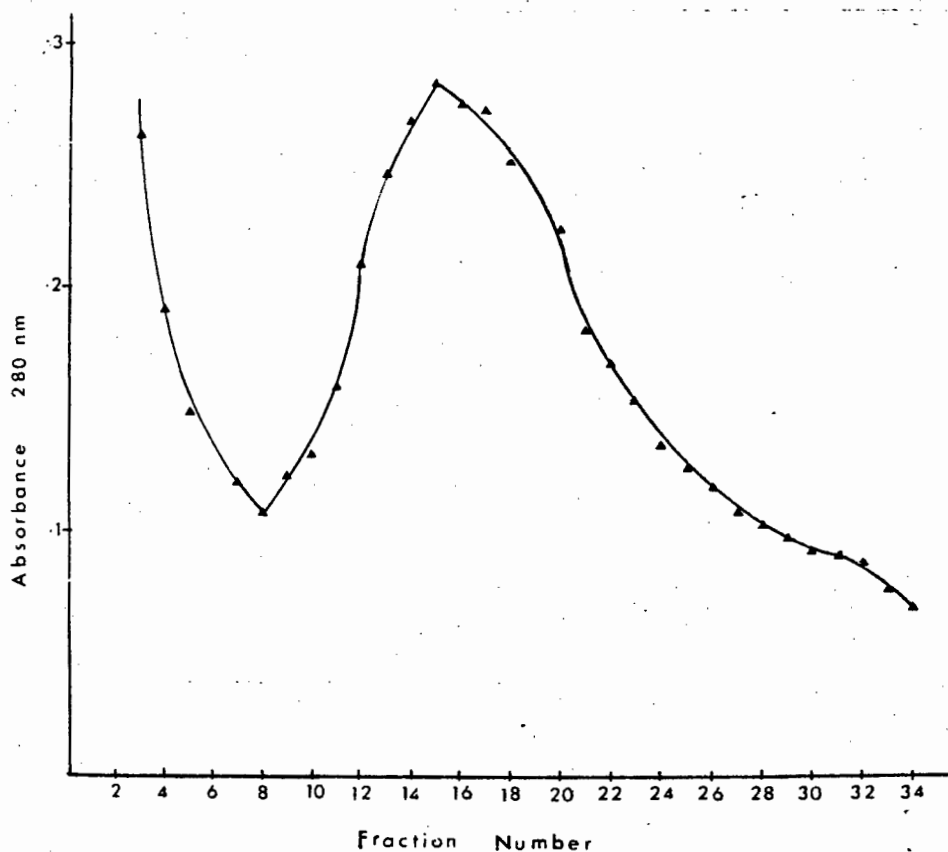


Fig.9. Fractionation pattern from a zone electrophoresis column. IgM (10mg) obtained after gel filtration, was electrophoresed for 3 days in a sucrose gradient prepared in borate buffer, pH 8.6. Fractions (2ml) were collected manually; the absorbance at 280nm was measured on a Unicam spectrophotometer.



Fig.10 Immunoelectrophoretic pattern of the IgM preparation after zone electrophoresis. Goat anti rabbit<sup>IgM</sup> antiserum diluted  $\frac{1}{2}$  was placed in the upper trough, and goat antiserum to rabbit serum proteins diluted  $\frac{1}{2}$  was placed in the lower trough. The well contained the IgM preparation obtained after zone electrophoresis.

increasing the ZE time from 3 days to 4 or 5 days. These attempts were unsuccessful owing to the considerable denaturation of IgM which occurred during ZE.

All IgM isolated for the work in this thesis was prepared by a combination of gel filtration method (See Fig 7) and ZE at pH 8.6 for 3 days, unless otherwise stated.

b. Purification of IgM from serum by polyethyleneglycol (PEG) precipitation.

Because of the inadequate purification of the IgM by the method outlined in the preceding section an alternative method was tested. Immunoglobulins were precipitated from rabbit serum by 4% PEG (Polson et al 1964), and were then separated by gel filtration on Sephadex G200 (see flow diagram in Fig 11).

When the IgM sample collected from the Sephadex G200 column was tested by immunoelectrophoresis the preparation appeared pure (Fig 12). The purity, however, was not confirmed by the immunization of a goat or rats, since insufficient IgM was obtained.

The rabbit serum (5ml) used in the precipitation experiment was collected from rabbits at the peak of infection and was estimated from later work (See table 5) to contain between 25mg and 30 mg IgM. A total of 0.7mg IgM was obtained after gel filtration. (See flow diagram in Fig. 11).

Figure 11. Procedure for the isolation of rabbit IgM from the serum of trypanosome infected rabbits, by the PEG precipitation method.

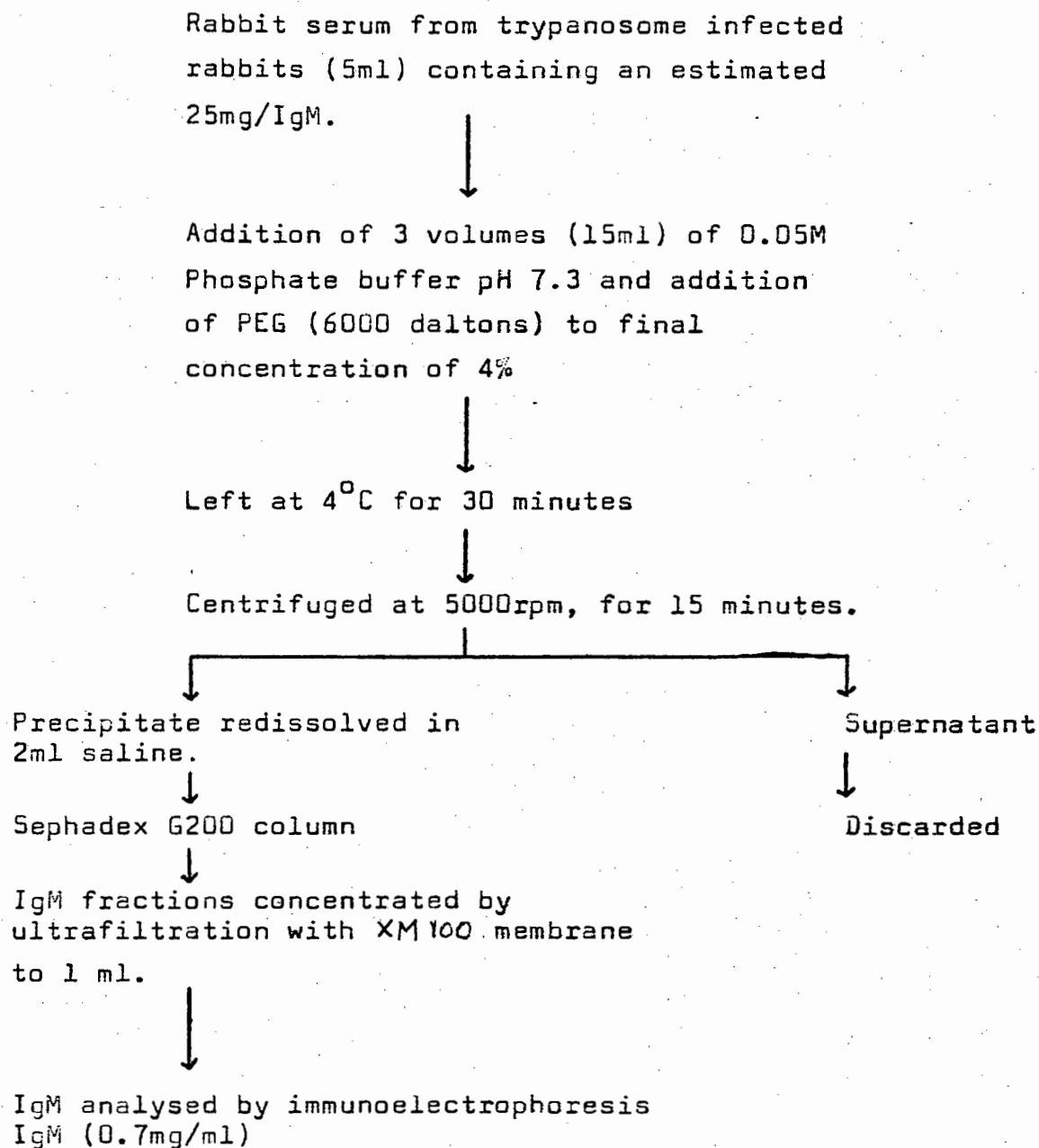






Fig. 12 Immunoelectrophoretic pattern of the IgM preparation obtained by PEG precipitation of rabbit serum. Upper trough: goat anti rabbit IgM antiserum  $\frac{1}{2}$  (Cappel Lab.Inc., Downingtown, P.A.,); Lower trough: goat anti rabbit IgM antiserum (undiluted); Upper well: rabbit IgM after gel filtration; Centre well: rabbit IgM obtained after PEG precipitation of rabbit serum and gel filtration (see Fig.11). No  $\alpha_2$  macroglobulin precipitin band can be seen. Lower well: serum from a trypanosome infected rabbit. The precipitin band formed by the  $\alpha_2$  macroglobulin is clearly visible (see arrow 4).  
 Arrow 1: IgG; Arrow 2: IgM; Arrow 3:  $\beta$  lipoprotein.

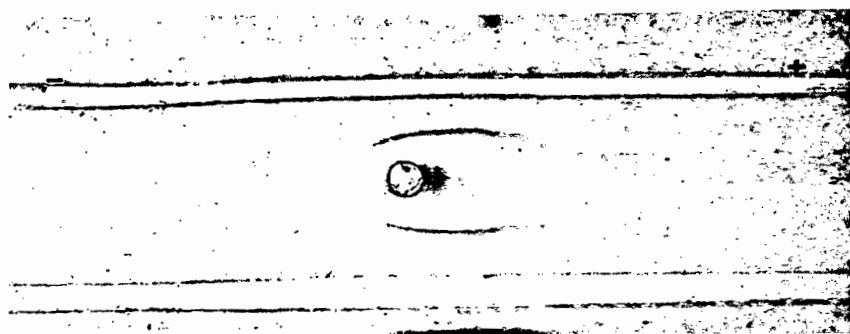


Fig. 14. Immunoelectrophoretic pattern of the IgM preparation purified by PEG precipitation. (See Fig. 13).  
 Upper trough: goat antiserum to rabbit serum proteins diluted  $\frac{1}{2}$   
 Lower trough: goat anti rabbit IgM antiserum diluted  $\frac{1}{8}$ .

c. Purification of IgM from a gel filtration fraction by PEG precipitation.

In an attempt to increase the yield of purified IgM using the 4% PEG method, precipitation was performed on partially pure IgM prepared by gel filtration on Sephadex G200 (see flow diagram in Fig.13).

The IgM appeared pure by immunoelectrophoresis against goat anti rabbit serum (Fig 14), i.e. the  $\alpha_2$  macroglobulin concentration was below the level of detection. No immunization experiments were conducted.

A final IgM quantity of 0.7mg was obtained after PEG precipitation of 4.6mg partially pure IgM.

The methods outlined in Sections 1b and 1c were found to be unsuitable for use in the preparation of IgM standards, in view of the poor IgM yields.

With the assistance of Dr. A. Polson another method using a double 4% PEG precipitation step was tried in preliminary experiments. Immunoglobulins were precipitated from 30-50ml rabbit serum by two successive 4% PEG precipitation steps and were separated by ultra-centrifugation in a thin layer rotor (Polson 1971). This was followed by zone electrophoresis in a sucrose gradient at pH 8.6 for 3 days. Preliminary results from two experiments indicated that considerable denaturation of IgM had occurred at the centrifugation stage.

Figure 13.

Procedure for the purification of rabbit IgM obtained after gel filtration on a G200 column, by the PEG precipitation method.

10ml Partially pure IgM (2.3mg/ml prepared by gel filtration and ultrafiltration from 15ml serum from trypanosome infected rabbits.

2ml partially pure IgM  
to which was added 6ml 0.06M  
Phosphate buffer pH 7.3 and  
PEG to final conc. of 4%

8ml partially pure IgM

Stored at  $-18^{\circ}\text{C}$ .

Left at  $4^{\circ}\text{C}$  for 45 minutes

Centrifuged at 5000rpm for  
15 minutes.

Precipitate redissolved in  
0.5ml saline.

Supernatant discarded

IgM analysed by immunoelectrophoresis.

## 2. Purification of IgM<sub>S</sub> (Monomeric IgM).

In view of the difficulty in purifying sufficient 19S IgM of a suitable quality for antiserum production, attempts were made to purify and use monomeric IgM for this purpose. Furthermore 7S IgM was required as a standard for quantitative gel diffusion. Since  $\alpha_2$  macroglobulin and 7S IgM can be separated on the basis of size by gel filtration, it was thought that the production and isolation of IgM<sub>S</sub> (artificially produced monomeric IgM) would provide a suitable IgM purification method. This method, however, requires that the  $\alpha_2$  macroglobulin should remain stable in the presence of the reducing agents used to dissociate IgM. Three methods were used in attempts to reduce 19S IgM.

### a. Cysteine reduction of IgM

IgM (3mg) prepared by gel filtration and zone electrophoresis methods was reduced with 0.05M cysteine in 0.1M Tris buffer 8.6, at room temperature and then alkylated with 0.08M Iodoacetamide. The reduced sample was examined by analytical ultracentrifugation, and found to contain 4 components (Fig 15A & 15B). Two of the components were IgM aggregates present in approximate concentrations of 0.2mg/ml and disappeared from the schlieren patterns after a few minutes. The remaining components had sedimentation coefficients of 16.7 and 6.1, with the latter component present in the highest concentration. (Fig.15B).

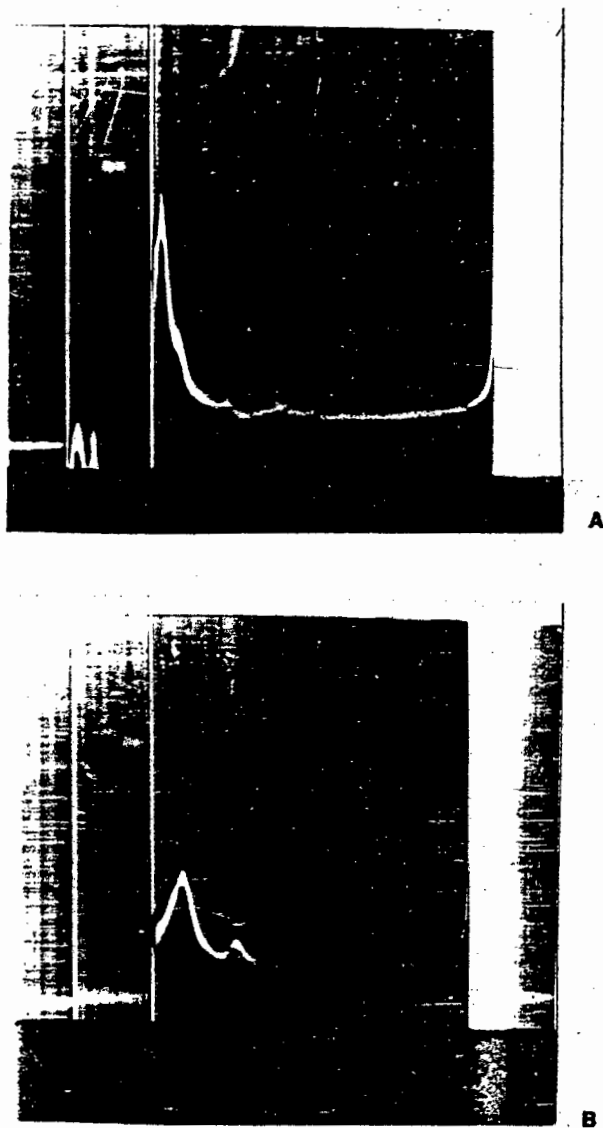


Fig.15. Ultracentrifugation patterns of purified IgM reduced with 0.05M cysteine, and alkylated with 0.08M iodoacetamide. Phase plate angle was  $60^{\circ}$ . A, IgM preparation immediately after reaching full speed (56,000 rpm); B, IgM preparation, 8 minutes after reaching 56,000 rpm.

Attempts were made to separate the reduction products on a Sephadex G200 column, but only a single peak was observed, when the absorption of the eluate was monitored at 280nm (Fig.16). The fractions of this peak were pooled (approximately 70ml), concentrated to 1ml by ultrafiltration and analysed by analytical ultracentrifugation. Only a small 19S schlieren peak was present. Presumably this was composed of reassociated monomers and/or unreduced IgM. Considerable IgM loss occurred during the gel filtration step. Purification by alternative methods, of the 7S IgM formed by cysteine reduction, was not tried.

b. Mercaptoethanol reduction of IgM

Rabbit IgM (2ml) at a concentration of 2mg/ml prepared by gel filtration and zone electrophoresis was reduced with an equal volume of 0.5M mercaptoethanol for 1 hour at 37°C. The reduction products were concentrated to 0.5ml by ultrafiltration, with an XM 50a Amicon membrane and analysed by analytical ultracentrifugation. Examination revealed a major component with a sedimentation coefficient of approximately 18S, and smaller amounts of more rapidly sedimenting material. An attempt was made to separate the components on a sucrose gradient, but poor separation was achieved.

c. Dithiothreitol reduction of purified IgM

Purified IgM (3mg/ml) was successfully reduced with 2mM dithiothreitol (DTT) and alkylated with 3mM iodoacetamide. An aliquot (0.15ml) of the reduced IgM was layered on each of 6 sucrose gradients.../

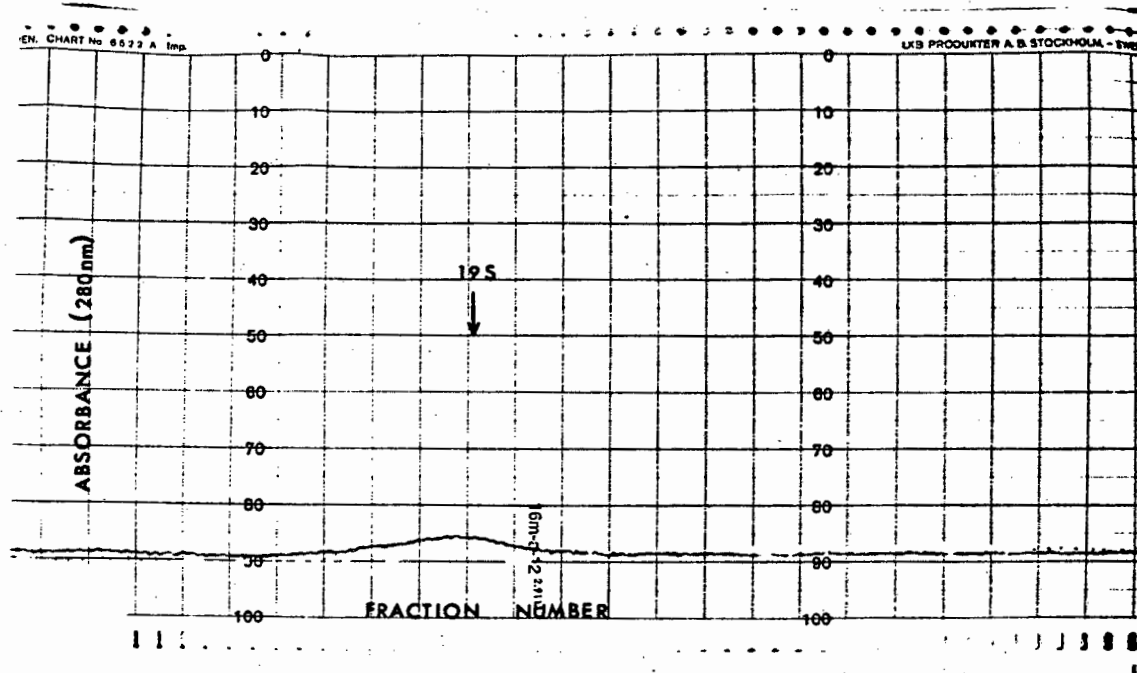


Fig. 16. Fractionation pattern of cysteine-reduced IgM after gel filtration on Sephadex G200. The column was eluted with 0.05M phosphate buffer pH 7.3, at a flow rate of 24ml/hr., and absorbance was monitored at 280nm. The position of the peak corresponded to that of the 19S components (See Fig.7).

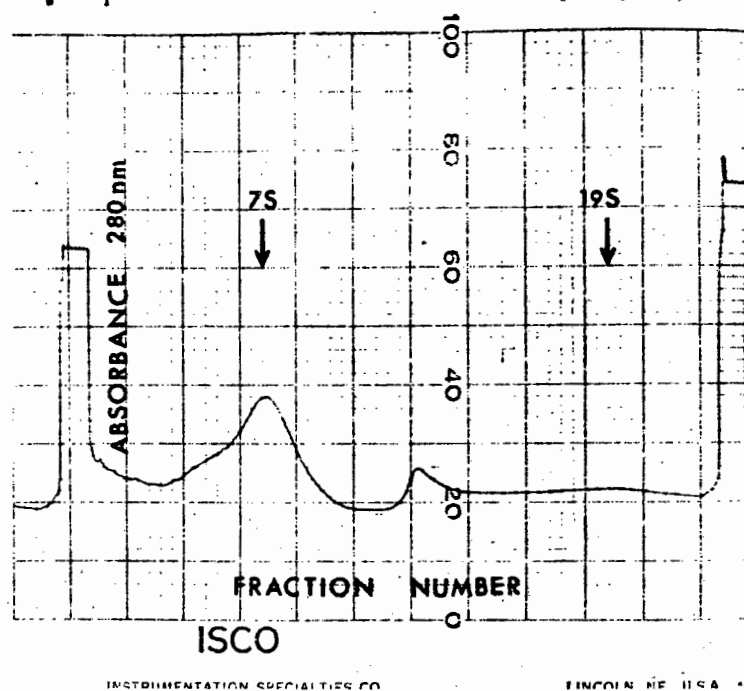


Fig. 17. Fractionation pattern of dithiothreitol-reduced IgM after sucrose density gradient centrifugation. The gradient was fractionated at 3ml/min with an Isco density gradient fractionator (Isco, Lincoln, Nebraska); the absorbance (280nm) was recorded at a chart speed of 150cm/hr.



gradients and centrifuged for 24 hours at 26,000 rpm. The density gradient fractionation pattern measured by absorbance at 280nm can be seen in Fig.17. The position of the oligomeric IgM corresponded to that of the 13.4S IgM produced by DTT reduction of serum (see section A, 2, d ).

Pooled fractions corresponding to the 7S and 13.4S components were dialysed overnight at 4°C, against normal saline. Both fractions were concentrated by ultrafiltration, the 7S fraction to 1ml and the 13.4S fraction to 0.6ml. Confirmation of the nature and purity of the 7S IgM was made by immunoelectrophoresis against both goat antiserum to rabbit serum proteins and goat anti-rabbit IgM serum (Fig 18). No precipitin bands were, however, observed on electrophoresis of the 13.4S fraction against both the goat antisera. The 7S fraction was subsequently used in the preparation of a calibration curve by quantitative gel diffusion.

d. DTT reduction of the 19S IgM present in serum.

Reduction and alkylation of 1ml of serum obtained from a rabbit at the peak of the infection was achieved. A schlieren pattern of the reduced serum showed both a 13.4S peak and more slowly sedimenting material, but no 19S peak. This indicated the likelihood of  $\alpha_2$  macroglobulin denaturation under the reduction conditions.

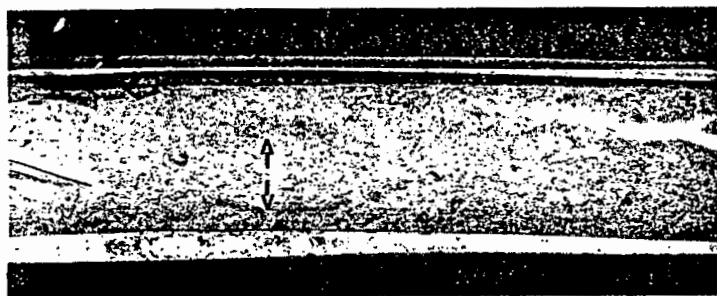


Fig. 18 Immunoelectrophoretic pattern of monomeric (7S) IgM (0.5mg/ml) (see arrows) produced by dithiothreitol reduction of purified IgM.

Upper trough, goat antiserum to rabbit serum proteins diluted  $\frac{1}{2}$   
Lower trough, goat anti rabbit IgM antiserum diluted  $\frac{1}{4}$ .

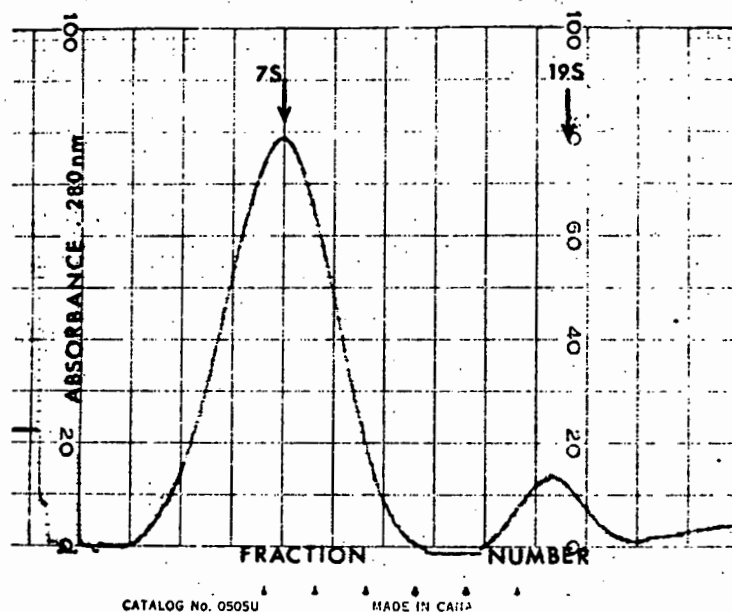


Fig.19. Fractionation pattern of 0.1ml serum, after sucrose density gradient centrifugation. The serum was obtained from <sup>rabbit</sup> 5, nine days after infection with *T. equiperdum*.

The gradient was fractionated at 3ml/min, and the absorbance (280nm) was recorded at a chart speed of 150cm/hr.

Reduced serum (0.1ml) was centrifuged on each of 3 sucrose gradients at 26,000 rpm, and the material located at the level of 19S components was collected. Despite concentration of this material to 0.2ml, no  $\alpha_2$  macroglobulin precipitin bands were observed by immunoelectrophoresis against goat antiserum to rabbit serum proteins. It is not known whether the denatured macroglobulin sedimented in the 13.4S, 7S or in an alternative position.

### 3. Fractionation of Serum by Density Gradient Centrifugation.

Both 7S and 19S serum fractions were required for IgM quantitation, anti trypanosome antibody assays and complement fixation tests. The fractions were prepared by layering 0.1ml of serum on each sucrose gradient, and centrifuging for 24 hours at 26000 rpm and at 4°C. After fractionation with an Isco fractionator the 7S and 19S fractions were dialysed against phosphate buffered saline 0.05M pH 7.3 (or normal saline if they were to be used for ultracentrifugation.), and were each finally concentrated to 1ml by ultrafiltration. In Fig.19 the analysis of a sucrose gradient, on which 0.1ml of the serum from rabbit 5 at day 9 was centrifuged, is shown.

The 19S fractions were analysed by immunoelectrophoresis to determine whether they were completely free of IgG (Fig.20). No IgG was detected but a faint precipitin line could be seen with  $\alpha_2$  macroglobulin.

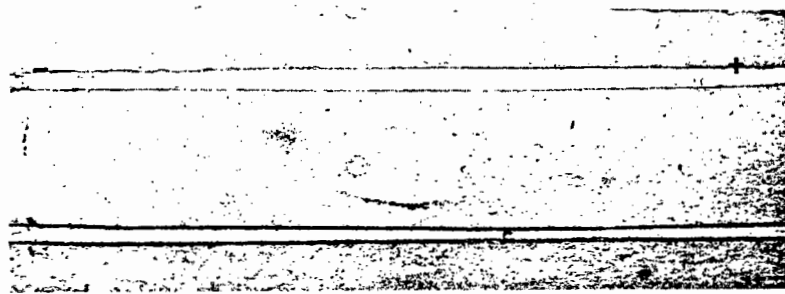


Fig.20. Immunoelectrophoretic pattern of the 195 fraction obtained after density gradient centrifugation of 0.1ml serum. Both upper and lower troughs contained goat antiserum to rabbit serum proteins, at a dilution of  $\frac{1}{2}$  and undiluted respectively.

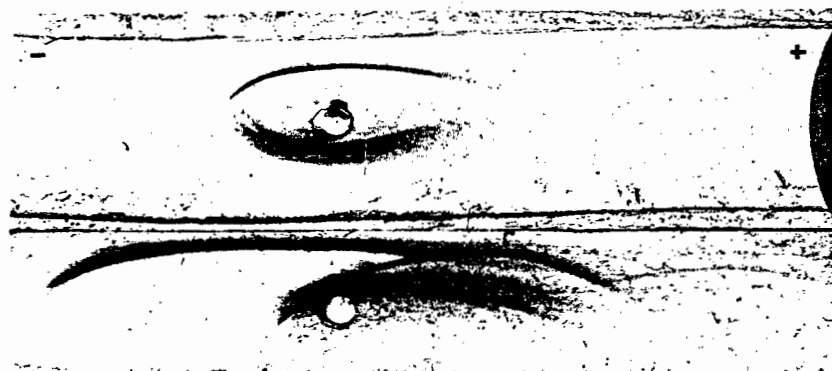


Fig. 21. Immunoelectrophoretic pattern of goat anti rabbit IgM anti-serum against normal rabbit serum and rabbit IgM. Upper well, IgM preparation after PEG precipitation and gel filtration (see Fig.12); Lower well, normal rabbit serum. The goat anti rabbit IgM anti-serum was used undiluted.

## B. PRODUCTION OF ANTISERA.

### 1. Preparation of Goat Anti Rabbit IgM Serum.

Goat anti rabbit IgM serum was produced by the immunization of a goat with IgM purified by zone electrophoresis. The reactivity of the antiserum obtained from the third bleed of the goat was analysed by immunoelectrophoresis against normal rabbit serum, (Fig. 21).

The antiserum reacted not only with rabbit IgM, but also with rabbit IgG,  $\alpha_2$  macroglobulin and  $\beta$  lipoprotein.

Attempts were therefore made to absorb those antibodies directed against  $\alpha_2$  macroglobulin,  $\beta$  lipoprotein and those cross-reacting with rabbit IgG in order to produce a  $\mu$  chain specific antiserum.

#### a) Absorption of the antiserum with rabbit IgG.

The goat antiserum was absorbed for 1 hour at room temperature with rabbit IgG at a concentration of 1,5mg/ml, and was retested by immunoelectrophoresis to determine whether it still reacted with IgG and IgM. The IgG absorbed the antibodies to the light chains, but a precipitin band was still formed with IgM (Fig. 22).

The goat antiserum absorbed with IgG was used in quantitative gel diffusion tests at a dilution of  $1/4$ . At this dilution the antiserum acted as a monospecific IgM antiserum, since all the  $\alpha_2$  macroglobulin and  $\beta$  lipoprotein antibodies had been diluted out. In rocket electrophoresis experiments.../



Fig. 22. Immunoelectrophoretic pattern of the goat anti rabbit IgM antiserum absorbed with rabbit IgG.

Upper trough: goat anti rabbit IgM antiserum.

Lower trough: goat anti rabbit IgM antiserum absorbed with IgG.

Centre well: rabbit IgG and rabbit IgM.

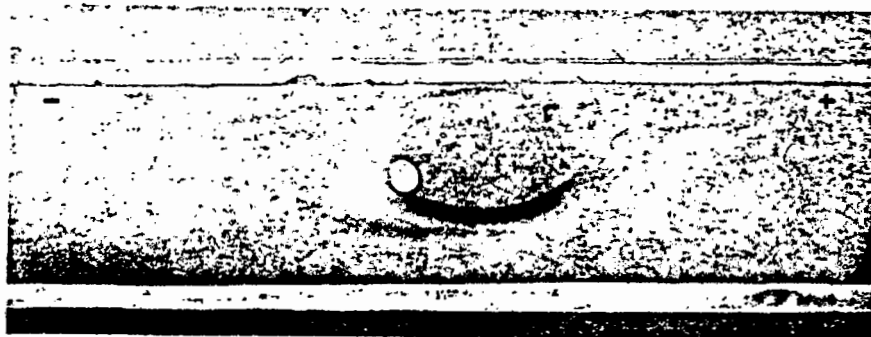


Fig. 23. Immunoelectrophoretic pattern of the goat anti rabbit IgM antiserum absorbed with rabbit neonatal serum.

Upper trough: goat anti rabbit IgM antiserum absorbed with neonatal serum.

Lower trough: goat anti rabbit IgM antiserum.

Centre well: partially pure rabbit IgM.



Fig. 24 Immunoelectrophoretic pattern of rabbit neonatal serum.

Top and bottom wells: purified IgM.

Centre well: neonatal rabbit serum (undiluted).

The upper trough was filled with goat anti serum to rabbit serum proteins diluted  $\frac{1}{2}$ , and the lower trough with goat anti rabbit IgM antiserum (undiluted).

c) Absorption of the goat anti rabbit IgM serum with dialysed NRS.

When normal rabbit serum (NRS) is dialysed against distilled water for three days at 4°C, the IgM precipitates out and may be removed by centrifugation, (Deutsch & Morton 1958). Providing all the IgM could be removed without removing the  $\alpha_2$  macroglobulin from the supernatant, it was thought that dialysed NRS could be used to absorb the anti  $\alpha_2$  macroglobulin antibodies and the anti  $\beta$  lipoprotein antibodies from the antiserum. Equal volumes of dialysed NRS and goat antiserum were mixed at room temperature for 1 hour and the absorbed antiserum tested against IgM by immunoelectrophoresis. Results indicated inadequate removal of the IgM from the NRS, since all the anti IgM antibodies were absorbed from the goat antiserum ie. there was no precipitin band formed against IgM.

d) Attempts to isolate  $\alpha_2$  macroglobulin for absorption experiments.

Attempts were made to purify  $\alpha_2$  macroglobulin from 10ml of rabbit serum for absorption experiments, by dialysis against 0.12mM acetate buffer pH 5.4 (Brown et al 1954). From IE studies it was found that IgM and small quantities of  $\alpha_2$  macroglobulin were precipitated during the dialysis. Absorption of the antiserum with the precipitate was not carried out.



## 2. Preparation of Goat Anti Rabbit Serum.

Goat anti rabbit serum was produced by immunizing a goat with normal rabbit serum proteins. The antiserum was found to react with at least 20 serum proteins by IE, and in this technique was thereafter used at dilutions from  $\frac{1}{2}$  to  $\frac{1}{16}$ . The antiserum was used at a dilution of  $\frac{1}{256}$  in rocket IE experiments.

## 3. Preparation of Goat Anti Rabbit IgG Serum.

The goat antiserum produced by injection of 14mg of rabbit IgG was absorbed with rabbit IgM at a concentration of 1.25mg/ml serum. After absorption the antiserum was still not specific for rabbit IgG, precipitin reactions also occurring with glycoproteins and Transferrin. However, the antiserum was used at a dilution of  $\frac{1}{8}$  in gel diffusion tests, at which only IgG precipitin bands were visible.

## C. QUANTITATION OF RABBIT IgM.

### I. Determination of IgM Concentrations by Quantitative Immuno-electrophoresis.

The concentration of IgM in the serum of two rabbits infected with T. equiperdum was measured by quantitative immuno-electrophoresis. Twofold dilutions of the sera of the rabbits were electrophoresed in 1% agarose in Tris buffer pH 8.1, 0.025M for  $1\frac{1}{2}$  hours. Precipitin bands formed against goat anti rabbit IgM serum ( $\frac{1}{4}$  dilution) were allowed to develop overnight. The slides were washed, and stained with Coomassie R250 stain.

Results indicated an increase in the IgM concentration from 1.5mg/ml at day 3 to a maximum of 6mg/ml at the peak of the infection, the concentration decreasing thereafter to 2mg/ml. In view of the large inherent error in the technique, estimated to be at least 50%, these results were only considered as preliminary quantitative indications of increased IgM concentrations at the peak of the infection. More accurate measurements by quantitative gel diffusion, analytical ultracentrifugation and rocket IE were then conducted.

2. Determination of IgM Concentrations by Gel Diffusion, Ultracentrifugation and Rocket IE.

The concentration of a purified IgM (19S) standard, used in the gel diffusion calibration curve, was determined by measuring the area under the schlieren peak, and by comparing it with the area obtained when a standard bovine serum albumin preparation was ultracentrifuged. The 19S IgM calibration curve was then prepared by gel diffusion of twofold dilutions of the purified IgM against a constant goat anti rabbit IgM antiserum at a dilution of  $1/4$  (Fig. 25A & 25B.). The IgM concentrations in the standard curve are, therefore, relative to the original IgM concentration which was determined by analytical ultracentrifugation.

IgM concentrations in the sera of a total of 6 infected rabbits were determined by both gel diffusion and by analytical ultracentrifugation. Initially the IgM concentrations in the sera and 19S fractions of 3 rabbits (Rabbits 1-3) were measured at 3-5 day intervals, starting from the day of inoculation. It is important to note.../

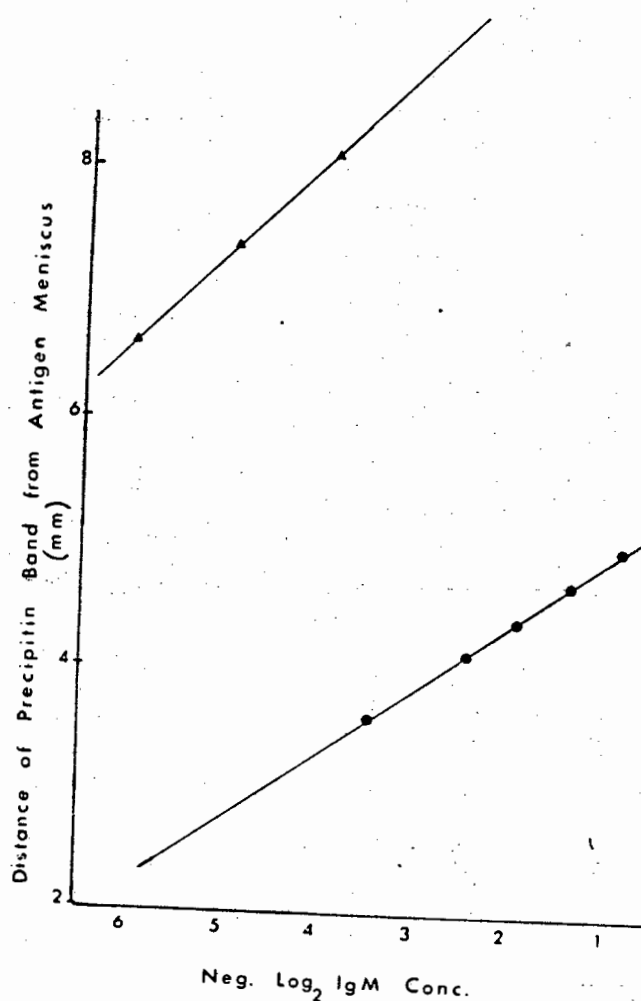


Fig. 25A. Quantitative double immunodiffusion with rabbit IgM.

Plot of distance of precipitin band from antigen meniscus against negative logarithm of IgM (7S, 19S) concentration. IgM preparations (8mg/ml) and goat anti rabbit IgM antiserum diluted  $\frac{1}{4}$  were used for the tests. Each point is the means of duplicate determinations.

●—● 19S IgM

▲—▲ 7S IgM

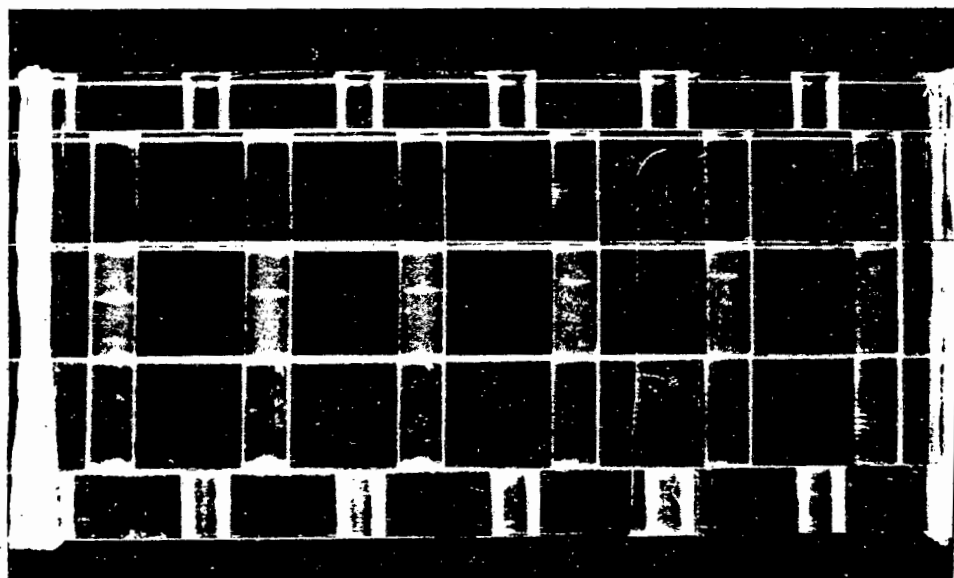


Fig. 25 B. Quantitative double immunodiffusion with rabbit IgM (19S). The top section of the apparatus contained a series of twofold dilutions of purified IgM; the middle section contained 0.75% agarose and the bottom holes contained goat anti rabbit IgM antiserum diluted  $\frac{1}{4}$ . Band position is a linear function of the logarithm of the ratio of antigen to antibody concentration.

to note that these rabbit sera had been stored at  $-18^{\circ}\text{C}$  for 1-2 months prior to the IgM determinations being performed. Results obtained from these three rabbits by quantitative gel diffusion and analytical ultracentrifugation are presented in Table 3.

The IgM concentrations increased from an average of 1.1mg/ml at day 0 to an average concentration of 16.0mg/ml after day 17 when measured by gel diffusion, and from 1.4mg/ml to 7.2mg/ml when determined by measuring the areas under the schlieren peaks. Thus there was little similarity between the IgM concentrations determined by the two methods at the peak of the infection, particularly as the concentration of IgM was overestimated due to the presence of  $\alpha_2$  macroglobulin in the 19S schlieren peaks. The agreement between the IgM concentrations in the sera and 19S fractions of rabbits 1-3 when measured by gel diffusion was also poor. In fact the 19S fractions in rabbits 1 and 3 frequently had higher IgM concentrations than the corresponding sera, despite the IgM losses that occurred during the fractionation procedures.

A likely explanation for the general lack of agreement between the IgM concentrations in the sera and 19S fractions determined by quantitative gel diffusion and ultracentrifugation, was the presence of IgM degradation products, existing in differing amounts in the various sera and 19S fractions. IgM degradation may have occurred, both during the fractionation and the storage of the sera. The products so formed would diffuse far more quickly.../

TABLE 3

Concentrations of IgM and 19S serum components  
in I. equiperdum infected rabbits (1-3)

	Days after infection.	IgM (mg/ml) determined by gel diffusion <sup>a</sup>		19S components (mg/ml) <sup>b</sup>	
		Serum	19S	Serum	19S
Rabbit 1	0	1.1		1.0	
	3	1.1		0.9	
	6	1.1		1.1	
	11	4.0		2.7	0.3
	13	8.0	5.1	4.0	1.5
	17	9.7	8.0	5.1	2.6
	21	14.8	19.3	8.7	3.0
	24	14.2	20.4	8.9	2.5
	27	10.2	8.0	6.2	1.9
Rabbit 2	0	0.6		1.7	
	3	1.4		1.9	
	6	1.6			
	11	4.0		3.7	1.0
	13	10.2	4.5	3.2	
	17	14.2	8.0	4.3	2.1
	21	10.8	8.5	4.7	2.8
	24	9.7	11.4	5.1	2.1
	27	8.0	4.0	5.4	1.3
Rabbit 3	0	1.7		1.4	
	3	2.1		1.6	
	6	1.5			
	11	4.5		1.9	0.6
	13	8.5		4.8	2.2
	17	13.1	c	6.4	2.9
	21	19.0		6.3	1.2
	24	14.5		6.8	1.2
	27	8.8		7.4	1.2

a Values are from duplicate measurements made in the apparatus depicted in Fig 25B, and are calculated from a standard calibration plot obtained with a purified IgM preparation.

b. Values are from triplicate measurements of areas under 19S schlieren peaks.

c. Values were too high to be determined accurately by gel diffusion.

more quickly than 19S IgM in the gel diffusion tests, so that falsely elevated IgM concentrations were determined when the band positions were compared with those of 19S IgM standards. This suggestion was supported by both the comparison of 7S and 19S IgM standard curves (Fig. 25A), and the closer agreement between the IgM concentrations determined by gel diffusion and ultracentrifugation in freshly obtained sera (Rabbits 4-6). The concentrations of 7S IgM (rabbits 1-3) were measured by gel diffusion with reference to the standard curve (Fig. 25A) prepared with monomeric IgM formed by DTT reduction of 19S IgM. The 7S IgM concentrations ranged from 1.3mg/ml at day 17 to a maximum of 1.9mg/ml at day 21. Since IgM (7S) precipitin bands were not formed by gel diffusion of the 7S fractions of rabbits 4-6 (diluted  $\frac{1}{2}$ ) it is likely that the 7S IgM in rabbits 1-3 was formed as a result of 19S IgM breakdown.

The concentration of IgM (19S) in the sera (rabbits 4-6) increased from 0.6mg/ml at day 0 to an average concentration of 5.3mg/ml at day 21 when measured by gel diffusion, and from 1.5mg/ml to 6.7mg/ml at day 21 when determined by analytical ultracentrifugation (see Table 4). There was a better correlation between the 19S IgM concentrations and the serum concentrations than in the previous set of results (Table 3), although there was still a considerable loss of IgM during fractionation. In a comparison of the IgM concentrations obtained by immunodiffusion and from measurements of the schlieren peaks, it must be noted that  $\alpha_2$  macroglobulin was present in the 19S schlieren peaks and that .../

TABLE 4

Concentrations of IgM and 19S serum components  
in T. Equiperdum infected rabbits (4-6)

	Days after Infection.	IgM (mg/ml) determined by gel diffusion <sup>a</sup>		19S components (mg/ml) <sup>b</sup>	
		Serum	19S	Serum	19S
Rabbit 4	0	0.6	<0.5	1.4	0.8
	9	2.3	1.0	3.4	1.4
	21	5.3	3.1	6.3	3.9
Rabbit 5	0	0.6	<0.5	1.7	1.2
	9	2.3	1.6	3.4	2.3
	21	5.7	3.8	6.7	4.1
Rabbit 6	0	0.7	<0.5	1.4	<0.8
	9	2.0	1.1	3.2	1.2
	21	4.8	2.1	7.2	2.3

a. Values are from duplicate measurements made in the apparatus depicted in Fig. 25B, and are calculated from a standard calibration plot obtained with a purified IgM preparation.

b. Values are from triplicate measurements of areas under 19S schlieren peaks.



and that this resulted in an overestimation of IgM concentrations by the ultracentrifugation method.

Attempts were made to reduce the 19S IgM in rabbit serum with DTT and to measure the  $\alpha_2$  macroglobulin content by analytical centrifugation. DTT was, however, thought to have reduced the  $\alpha_2$  macroglobulin since on analysis of the reduced serum, no 19S peak was evident.

Fig. 26 shows the changes in the levels of 19S components calculated from the ultracentrifuge diagrams in rabbits 1-6. The concentrations of the 19S components increased after day 6 to reach a maximum at day 24.

The IgM concentrations in the sera of rabbits 4-6 were also measured by rocket IE. A calibration curve was constructed by measuring the peak heights of 6 IgM standards (0.5 - 3 mg/ml), after electrophoresis for 16 hours in 1% agarose, containing a final dilution of  $1/256$  of goat anti rabbit IgM serum (Fig. 27A & 27B).

Duplicate runs were then carried out on  $1/2$  and  $1/4$  dilutions of the sera, and the heights of the peaks compared with those of the IgM standards (Fig 28). The results obtained by rocket IE are compared with those of gel diffusion. (See Table 5).

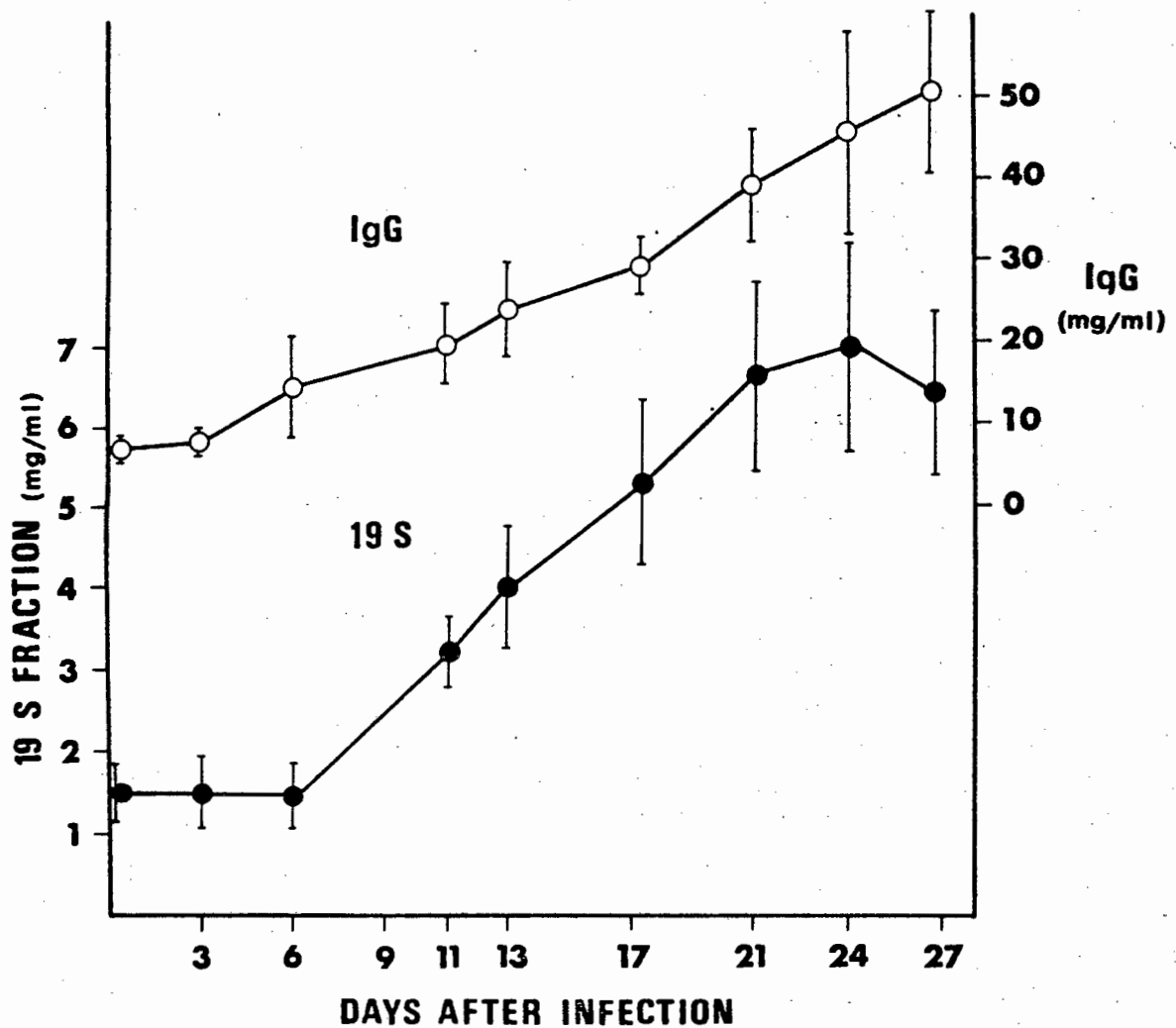


Fig. 26. Concentration of IgG and 19 S serum fraction during T. equiperdum infection in the rabbit. Each point is the mean of 3-6 measurements on different animals  $\pm$  standard deviation. The amount of IgG was obtained from double immunodiffusion tests and the 19 S fraction by measuring the area under schlieren peaks.

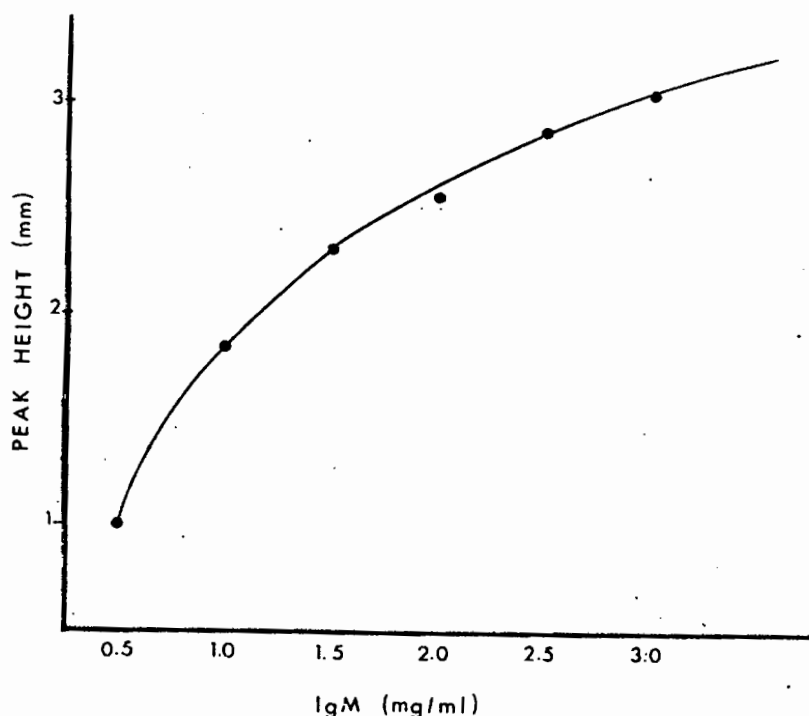


Fig. 27A. Standard curve of rabbit IgM (mg/ml) against peak height (mm) determined by rocket immunoelectrophoresis at 2 v/cm overnight. Goat anti rabbit IgM antiserum was used at a final dilution of  $1/256$ . Each point is the mean of duplicate determinations. (See Fig. 27B)

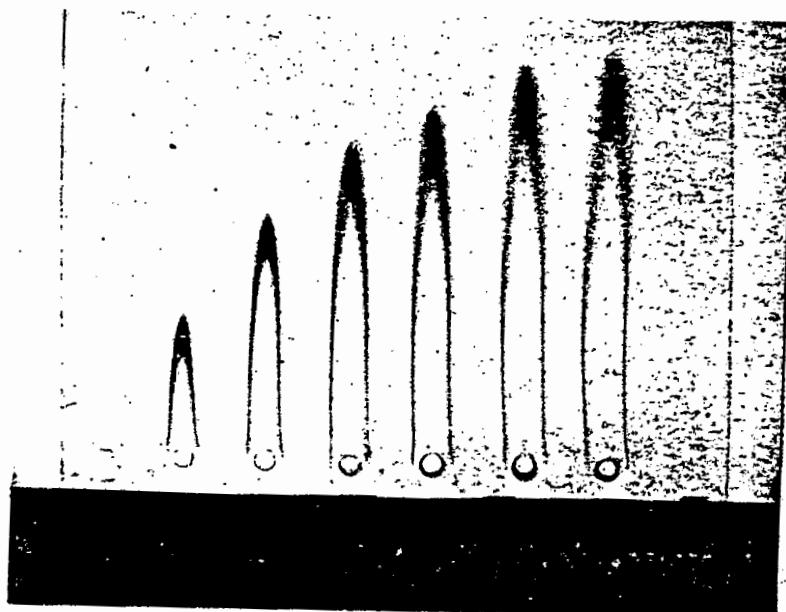


Fig. 27B. Rocket immunoelectrophoresis performed at pH 8.8 in 1% agarose. IgM standards (0.5 - 3.0 mg/ml) were electrophoresed against goat anti rabbit IgM antiserum at a final dilution of  $1/256$ .

TABLE 5.  
IgM levels in sera from rabbits infected with Trypanosoma equiperdum.

	Day after infection	IgM (mg/ml) determined by double diffusion <sup>a</sup>	IgM (mg/ml) determined by rocket immuno- electrophoresis <sup>b</sup>	Average IgM (mg/ml)	19S fraction (mg/ml) <sup>c</sup>	% IgM in 19S fraction.
Rabbit 1	0	0.6	0.7	0.65	1.7	38
	9	2.3	2.7	2.5	3.4	73
	21	5.7	7.3	6.5	6.7	97
Rabbit 2	0	0.6	0.3	0.45	1.4	32
	9	2.3	1.9	2.1	3.4	61
	21	5.3	5.1	5.2	6.3	82
Rabbit 3	0	0.7	0.5	0.6	1.4	43
	9	2.0	2.1	2.05	3.2	64
	21	4.8	7.2	6.0	7.2	83

a. Values are from duplicate measurements made in the apparatus depicted in Fig. 258, and are calculated from standard calibration plots obtained with a purified IgM preparation.

b. Values are from triplicate measurements. The relatively small amounts of monomeric IgM detected in the 7 S fraction by this technique were ignored.

c. Values are from duplicate measurements of areas under 19S schlieren peaks.

When the amount of IgM was expressed as a % of the total protein present in the 19S peaks, the relative proportion of IgM increased with time. These results agree with previous findings of Klein et al (1970), namely that in T. equiperdum infected rabbits the concentration of  $\alpha_2$  macroglobulin remains constant, and that in normal rabbits, IgM accounts for about 38% of the material present in the 19S peak. The results in Table 5 correspond to an approximate tenfold IgM increase, whereas Klein et al (1970) reported a sixfold increase of IgM.

The concentration of monomeric IgM in the 7S fractions of rabbits 4-6 was estimated by rocket immunoelectrophoresis using reduced IgM as a standard. At the peak of macroglobulinaemia about 0.5 - 1.0mg/ml of monomeric IgM could be detected, but because of the difference in electrophoretic mobility between native and reduced monomers of IgM (Hansson & Laurell 1970), no attempt was made to determine accurately the amount of 7S IgM present in the rabbit sera.

### 3. Attempts to Quantitate IgM by Laurell Electrophoresis.

Attempts were also made to quantitate the 19S IgM in the rabbit sera by Laurell electrophoresis.

Serum drawn from a rabbit on the 21st day after infection with T. equiperdum was electrophoresed against goat anti rabbit serum at a dilution of  $1/256$  (See Fig.29). Satisfactory separation of the serum components was achieved, but the IgM peak was partly obscured by other precipitin peaks indicating the necessity of using a goat anti rabbit IgM serum.

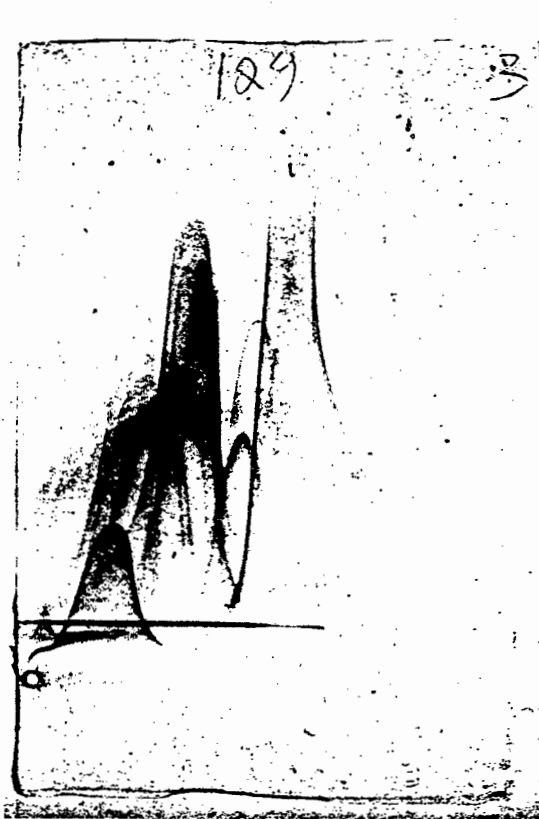


Fig. 29. Laurell electrophoresis of serum from a trypanosome infected rabbit, against goat anti rabbit serum antiserum at a dilution of  $1/256$ . Electrophoresis was performed in 1% agarose at pH 8.8 for 50 minutes at 6V/cm (first dimension), followed by electrophoresis at 1V/cm overnight. Precipitin peaks were stained with 0.5% Coomassie R250 stain.

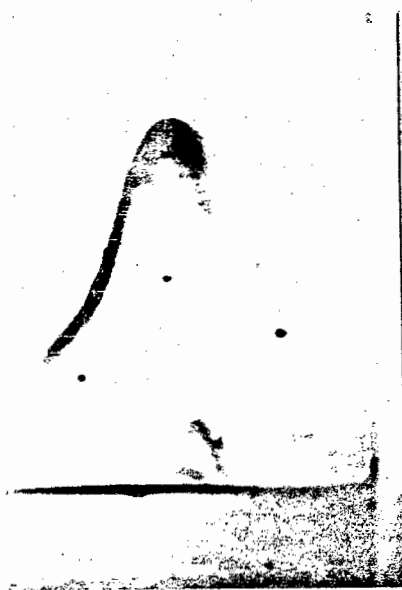


Fig. 30. Laurell electrophoresis of  $1.5 \mu\text{l}$  formalinized (0.1%) serum from a trypanosome infected rabbit, against goat anti rabbit IgM antiserum at a dilution of  $1/256$ . The precipitin peak was stained with 0.5% Coomassie R250 stain.

Purified IgM ( $1\mu\text{l}$  of  $2.76\text{mg/ml}$ ) was electrophoresed against a  $1/256$  dilution of goat anti rabbit IgM serum. The peak, although fairly clear, was incomplete, making area measurements difficult. Similarly faint IgM peaks were found on electrophoresis of rabbit serum against goat anti rabbit IgM serum.

In an effort to improve the clarity and size of the peaks, rabbit sera were formalinized with 0.01%, 0.05%, 0.1%, 0.2% and 2.0% formaldehyde, by dialysis against the formaldehyde solution overnight at room temperature (Polson 1976). Extremely indistinct and unsatisfactory IgM peaks were obtained (Fig.30).

Other attempts to improve the clarity of the peak included:

1. the use of Ponceau S, Amido Black, Procion Blue and 1% Coomassie R250 stains.
2. longer staining times of 40 and 60 minutes,
3. the use of precipitin brighteners eg 0.5% phosphotungstic acid, and 0.5% phosphomolybdic acid (Renn & Evans 1975) and
4. finally, the use of the commercially prepared anti rabbit IgM serum (Cappel Lab. Inc., D<sup>W</sup>ilmington, P.A.)

In addition the goat anti rabbit IgM serum, produced in the laboratory, was tested at dilutions of  $1/32$  to  $1/1024$ , of which the dilution of  $1/256$  was the most suitable.

The most satisfactory IgM peaks were obtained with the use of the precipitin brightener - 0.5% phosphotungstic acid (PTA) together with 0.5% Coomassie R250 stain (Fig.31).

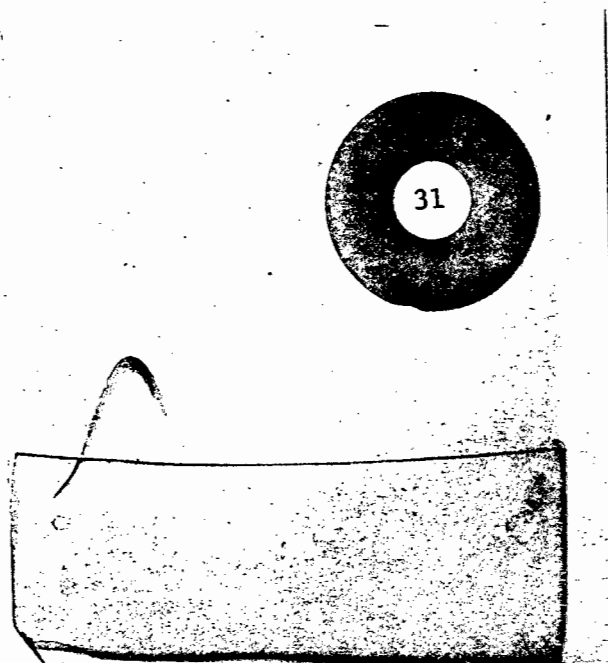


Fig. 31. Laurell electrophoresis of rabbit IgM (5mg/ml) against goat anti rabbit IgM antiserum at a dilution of  $1/256$ . The precipitin peak was brightened with 0.5% phosphotungstic acid and stained with 0.5% Coomassie R250 stain.



An IgM calibration curve was prepared using purified IgM at concentrations of 2-5mg/ml, using agarose containing goat anti rabbit IgM serum at a final dilution of  $1/256$ . The areas of the peaks were measured by determining the height of the peak from the well, and the width at a position of half the height (Weeke 1973b). This method was not very accurate, however, because of the formation of incomplete IgM peaks, and the resultant difficulty in measuring them. The IgM concentrations in the sera of rabbits 4-6 were then determined (see Table 6) by comparing the areas of the peaks obtained (Fig.32) with those of the IgM standards (Fig. 31 - only one standard is shown.)

If one compares the results obtained by Laurell electrophoresis with those obtained by rocket electrophoresis (see Tables 5 and 6), it is clear that large discrepancies exist between the IgM concentrations. The most likely explanation for those discrepancies is the inaccuracy of the area measurements, in Laurell electrophoresis, caused by the formation of incomplete IgM peaks, and for this reason it was decided that Laurell electrophoresis was unsuitable for IgM determinations.

It had been hoped that 7S IgM peaks would be visible by Laurell electrophoresis of serum from trypanosome infected rabbits; 7S IgM differs in electrophoretic mobility from 19S IgM and should form a separate peak. Unfortunately no 7S IgM peaks were visible on electrophoresis of the sera but specific attempts to detect 7S IgM were not made.

#### D. QUANTITATION OF IgG IN RABBIT SERA.

IgG concentrations in the sera of trypanosome infected rabbits 1-3 were determined by quantitative gel diffusion using goat anti rabbit IgG serum at a dilution of  $1/8$ . An IgG calibration

curve.../

TABLE 6

Laurell precipitin peak area measurements, and IgM (19S) concentrations in the sera of T. equiperdum infected rabbits (4-6)

	Days after infection	Area (cm <sup>2</sup> ) a	IgM (mg/ml)
Rabbit 4	0	52.9	< 2.0
	9	127.0	3.0
	21	175.6	> 10.0
Rabbit 5	0	64.4	< 2.0
	9	124.8	2.8
	21	167.6	9.0
Rabbit 6	0	46.0	< 2.0
	9	112.5	< 2.0
	21	155.0	7.5

a. Values are from duplicate measurements of the areas under the precipitin peaks.

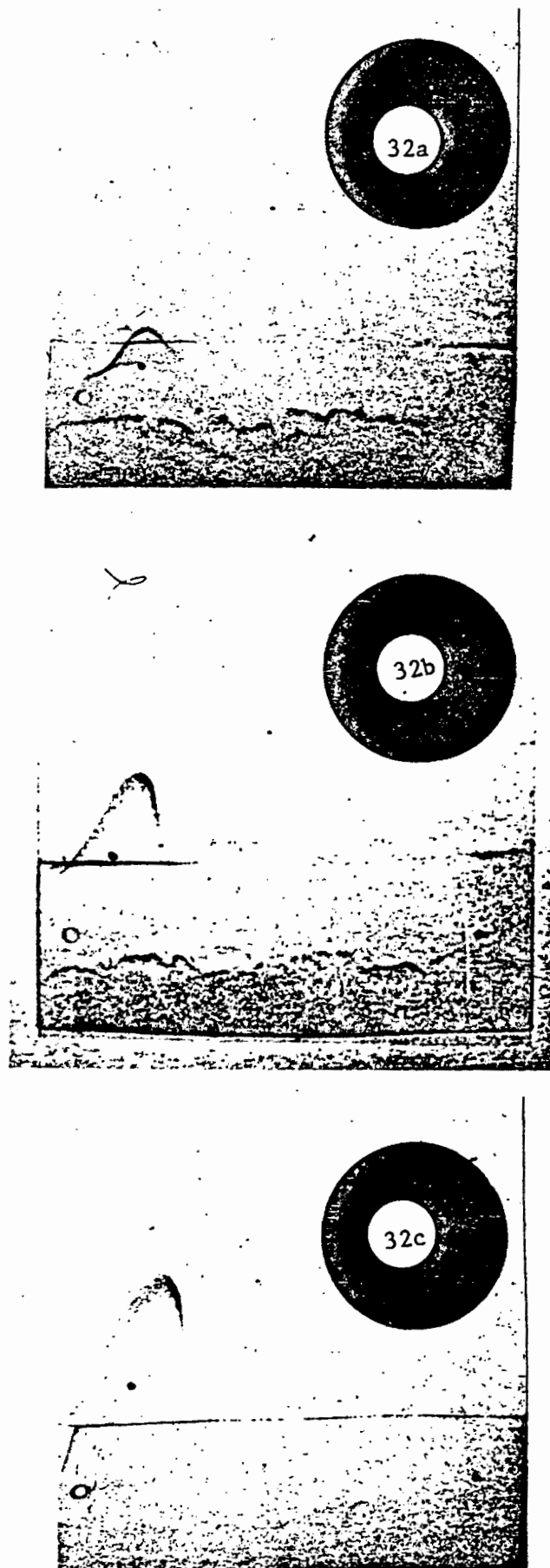


Fig. 32. Laurell electrophoresis of the sera obtained at days 0, 9, and 21 (Fig. 32A, 32B, 32C) from a trypanosome infected rabbit (rabbit 6) against goat anti rabbit IgM antiserum at a dilution of 1/256. The plate was treated with 0.5% phosphotungstic acid and the peaks were stained with 0.5% Coomassie R250 stain.

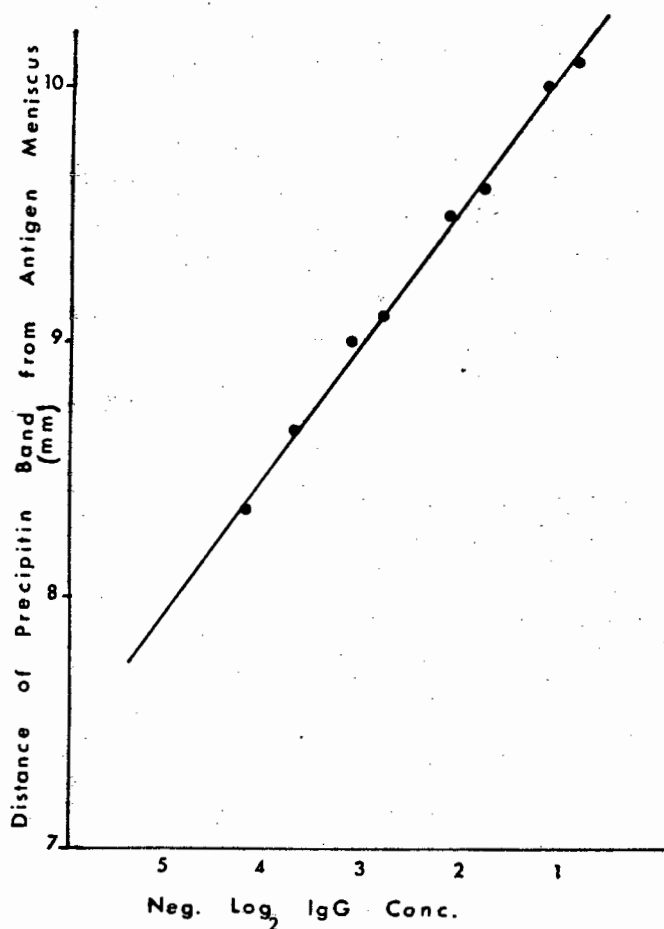


Fig. 33.A. Quantitative double immunodiffusion test with rabbit IgG. Plot of distance of precipitin band from antigen meniscus against negative logarithm of IgG concentration. IgG (Miles Lab. C.T.) was used at an initial concentration of 6.4 mg/ml; goat anti rabbit IgG antiserum was used at a dilution of  $1/8$ . Each point is the mean of duplicate determination.

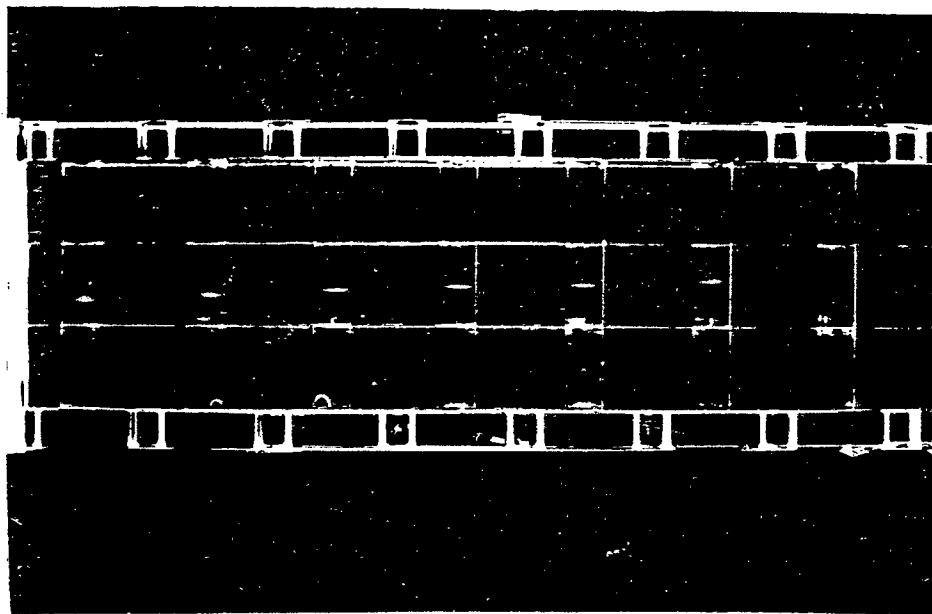


Fig. 33B. Quantitative double immunodiffusion with rabbit IgG. The top section of the apparatus contained twofold dilutions of IgG; the bottom section contained goat anti rabbit IgG antiserum diluted  $\frac{1}{8}$ . Precipitin bands were formed in the centre section which contained 0.75% agarose.

TABLE 7

IgG concentrations (mg/ml) in sera from rabbits (1-3)  
infected with Trypanosoma equiperdum.

Days after infection.	IgG (mg/ml) <sup>a</sup>		
	Rabbit 1	Rabbit 2	Rabbit 3
0	5.0	6.5	8.6
3	7.9	7.9	10.1
6		10.1	20.9
11	21.6	14.4	23.8
13	26.7	18.0	28.9
17	30.3	25.3	31.0
21	43.3	28.9	44.8
24	50.5	28.9	56.3
27	60.6	37.5	54.1

a. Values are from duplicate measurements obtained  
by gel diffusion.

TABLE 8.  
TESTS FOR RHEUMATOID FACTORS IN RABBIT SERA FROM T. EQUIPERDUM INFECTED ANIMALS AND IN  
HUMAN SERA FROM RHEUMATOID ARTHRITIS PATIENTS.

Antigen used for coating latex particles and sensitizing sheep red blood cells.	Reciprocals of titres of passive haemagglutination tests.		Reciprocals of titres of latex fixation tests. <sup>a</sup>	
	Rabbit Sera <sup>b</sup> .	Human serum from patient 1	Rabbit sera <sup>b</sup> .	Human serum from patient 2.
1. Rabbit IgG native 0.1 mg/ml .....	10	10240	10	2048
2. Rabbit IgG native 0.5 mg/ml .....	10	10240	10	2048
3. Rabbit IgG native 10.0 mg/ml .....	10	10240	10	512
4. Rabbit IgG heat denatured at 63°C, 10 minutes .....	10	10240		
5. Rabbit IgG heat denatured at 80°C, 10 minutes .....	10	10240		
6. Rabbit IgG denatured with 0.5m Urea .....	10	2560		
7. Rabbit IgG denatured with 1.0 m Urea .....	10	640		
8. Rabbit IgG denatured at pH 10 .....	10	10240		
9. Human Cohn Fraction II native 0.2 mg/ml .....	10	2560	10	512
10. Human Cohn Fraction II heat denatured at 63°C, 10 minutes .....	10	10240	10	512
11. Human Cohn Fraction II heat denatured at 80°C, 10 minutes .....	10	10240		

a) All sera were heat-inactivated at 56°C for 40 minutes.  
b) Eight rabbits were tested at four different times after infection.

F. THE NATURE AND QUANTITATION OF ANTI-TRYPANOSOME ANTIBODIES IN THE SERA OF T. EQUIPERDUM INFECTED RABBITS.

Anti-trypanosome antibody titres in the sera of trypanosome infected rabbits were initially determined by indirect immunofluorescence, using fluorescein-conjugated sheep anti-rabbit immunoglobulin serum at a dilution of  $1/8$ . The average anti trypanosome titres in the sera, and in the 19S and 7S serum fractions prepared by density gradient centrifugation, of rabbits 1-3 are shown in Fig 34.

Anti-trypanosome activity in the whole rabbit serum appeared 11 days after infection, with titres of  $1/160$  to  $1/320$ . Normal rabbit serum controls were consistently negative throughout the experiment, with titres of less than  $1/10$ . Immunofluorescent studies on the 19S and 7S serum fractions, however, showed a complete absence of anti-trypanosome activity in the 19S fractions, but titres up to  $1/80$  in the 7S fractions.

In order to determine whether the anti-trypanosome activity present in the 7S fractions was of IgM ( $\text{IgM}_N$ ) or IgG nature, indirect immunofluorescence was carried out using fluorescein conjugated goat anti rabbit  $\mu$  chain serum at a dilution of  $1/4$ . No anti-trypanosome activity was observed with this antiserum, indicating that the anti-trypanosome antibody activity was of the IgG type.



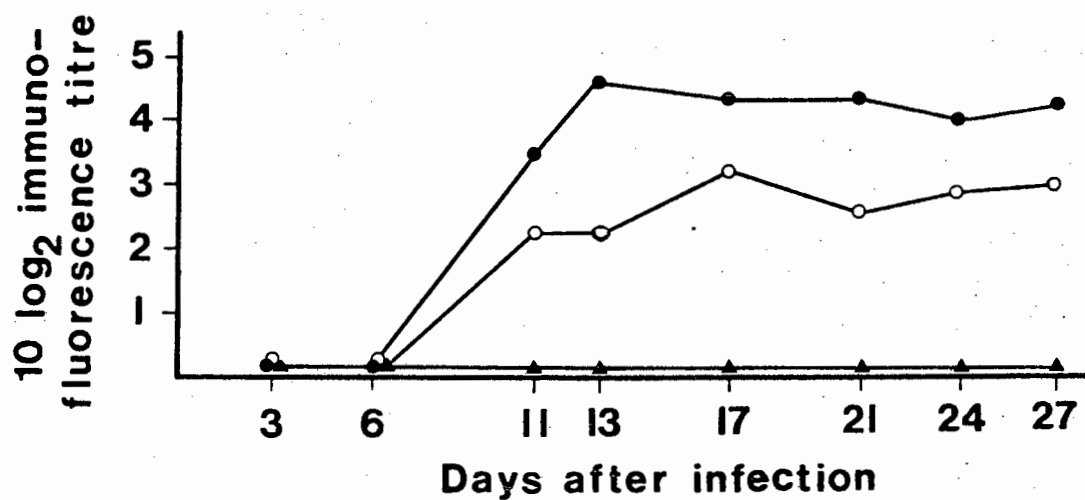


Fig. 34. Indirect immunofluorescence titres of anti-trypanosome activity in rabbits infected with T. equiperdum. A fluorescent sheep anti-rabbit immunoglobulin serum was used. Each point is the mean from three sera.

●-● Rabbit Serum ▲-▲ 19S fraction, ○-○ 7S fraction.

It was necessary to ensure that both the conjugated antisera used, could react with IgM antibodies, since the negative results obtained with the 19S fractions could otherwise be attributed to the inability of the antisera to bind to IgM. The reactivity of the fluorescent reagents with rabbit IgM was established by direct immunofluorescence on latex particles coated with IgM at a concentration of 1mg/ml.

Anti-trypanosome activity in 19S and 7S serum fractions of rabbits 1-3 was also measured by a more sensitive technique, - micro complement fixation. IgM (19S) antibody titres against whole trypanosomes reached a peak at day 13 with titres of  $1/1280$  and  $1/2560$ , and then decreased in all the animals tested. (See Fig. 35).

IgG (7S) antibodies appeared in a more erratic fashion (Fig 36) and different rabbits showed completely different patterns of increase and decrease of the IgG levels.

Confirmation of the anti-trypanosome activity in the 19S fractions was made by direct agglutination using purified trypanosomes as the antigen. IgM anti-trypanosome agglutination titres of  $1/640$  were observed at day 11, the titres decreasing thereafter, in an identical manner to those determined by complement fixation (See Fig 37).

Agglutination tests using whole infected blood as the antigen (Cunningham & Vickerman 1962) were unsatisfactory, owing to the difficulty in detecting agglutination.

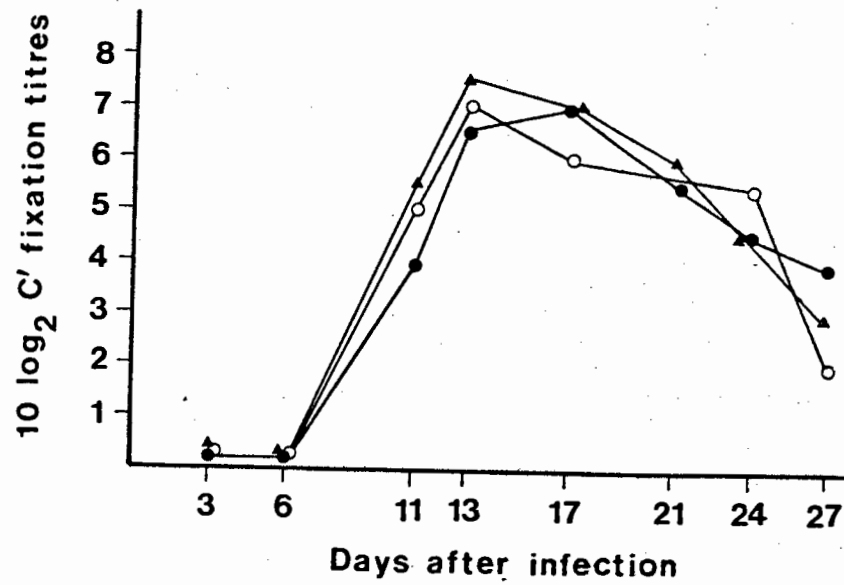


Fig. 35. Anti-trypanosome complement-fixation titres of 19 S serum fractions from rabbits infected with T. equiperdum. Individual results obtained from three different rabbits are shown. The antigen preparation used contained  $4 \times 10^6$  trypanosomes / ml.

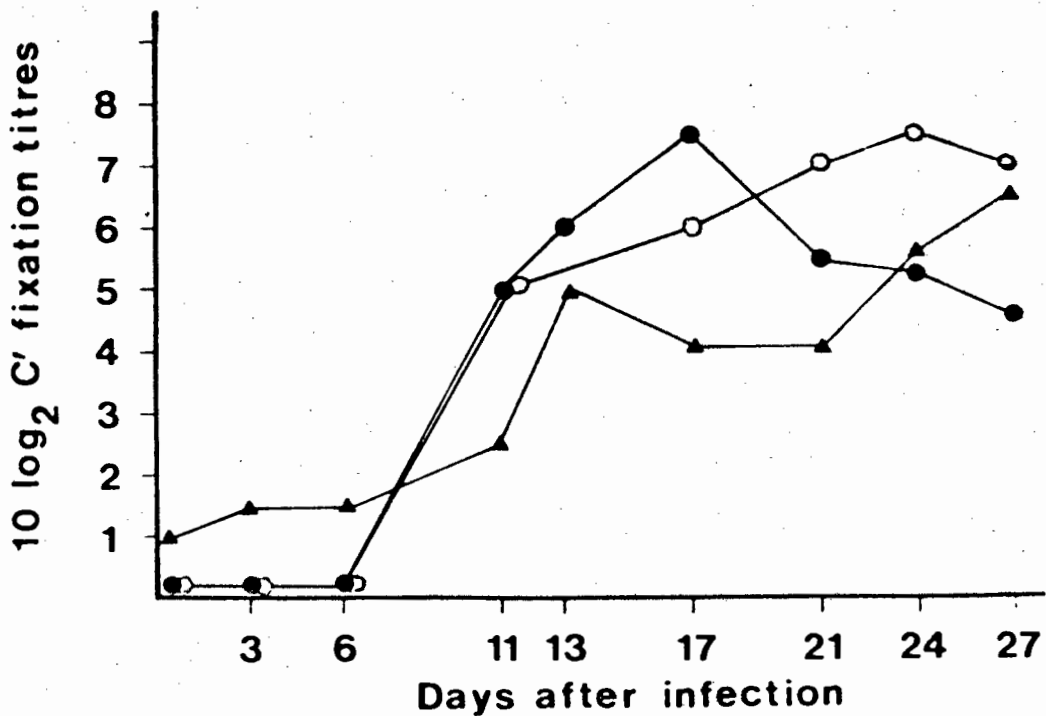


Fig. 36. Anti-trypanosome complement-fixation titres of the 7 S serum fractions from rabbits infected with T. equiperdum. Individual results obtained from three different rabbits are shown.

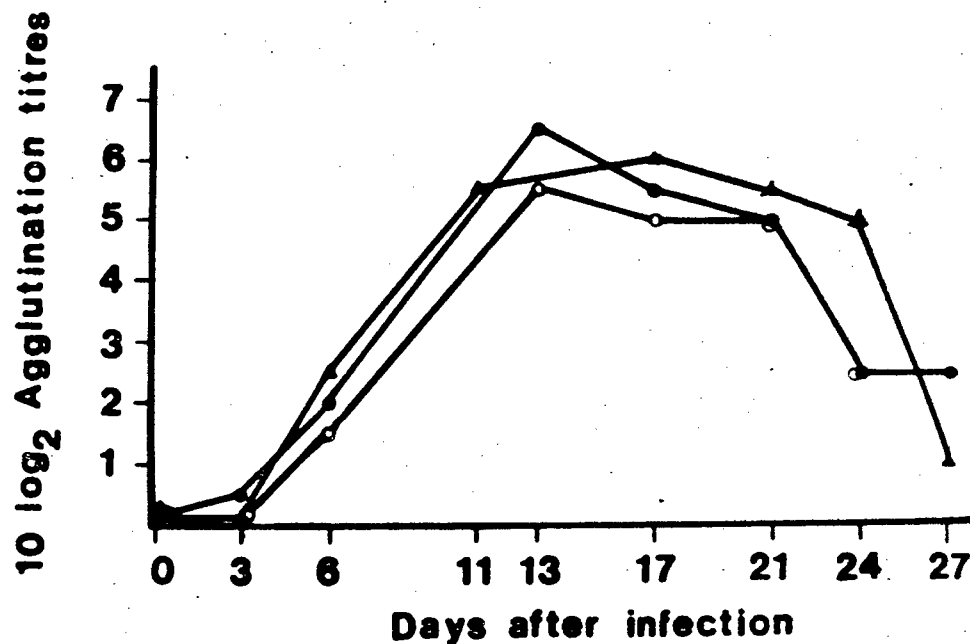


Fig. 37. Anti-trypanosome agglutination titres of the 19S serum fractions from rabbits infected with T. equiperdum. Individual results obtained from the same three rabbits as in Figs 34, 35, 36.

G. THE NATURE AND QUANTITATION OF TISSUE AUTOANTIBODIES  
IN TRYPANOSOME INFECTED RABBITS.

Autoantibodies to rabbit tissue antigens were detected in the sera of trypanosome infected rabbits 2 and 3 by micro complement fixation tests. The autoantibody titres of rabbits 2 and 3 to liver and kidney antigens obtained from both trypanosome-infected and normal rabbits are presented in Table 9. Titres are not available for the sera of rabbit 1 since this serum displayed anti-complementary activity.

No difference between the tissue antigens prepared from normal or infected rabbits could be detected in these tests.

The data in Table 9 are shown graphically in Fig 38. Antibody titres to liver antigen increased to a maximum between days 17-21 with titres of  $1/80$  -  $1/160$ , the titres thereafter decreasing until the death of the rabbits (See Fig. 38A). The anti-liver antibody activity was present exclusively in the 19S serum fractions and not in the 7S fractions, i.e. the auto-immune antibodies were of 19S IgM nature.

These results agree with previous findings (Mansfield & Kreier 1972a; Mackenzie & Boreham 1974).

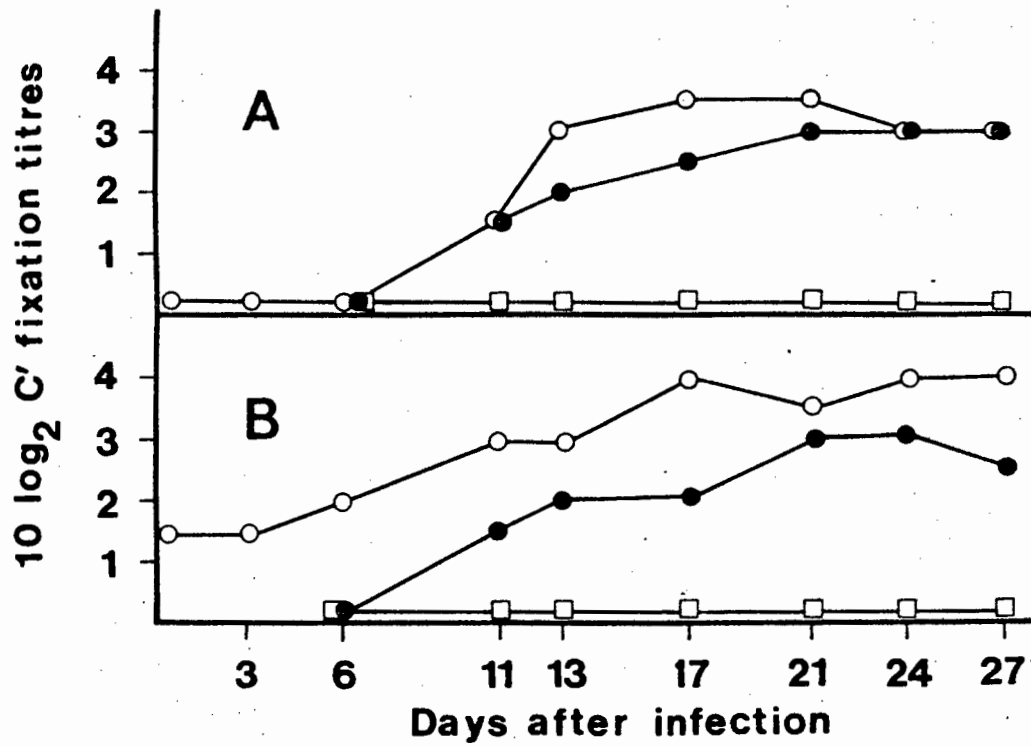


Fig. 38. Anti-tissue complement fixation titres of serum (o-o), 19S fraction (●-●) and 7S fraction (□-□) from two rabbits infected with *T. equiperdum*. Each point is the mean of two sets of duplicate measurements.

A: Anti-liver activity.

B: Anti-kidney activity.

The results obtained with kidney antigens were similar to those presented in Fig 38A, with the exception that the basal titre was  $1/20$  -  $1/40$  (Fig. 38B). The titres at the peak of the infection are, however, significantly higher than those found in T. congolense infected rabbits (Mansfield & Kreier 1972a) in which the maximum titres were  $1/32$ .

Normal rabbit serum controls had complement fixation titres of  $1/5$  with liver antigen, but of  $1/20$  with kidney antigen.

The autoantibody titres of rabbits 2 and 3 to heart antigens are shown in Table 10. Autoantibodies developed 6 - 11 days after infection, and rose to a maximum at the end of the infection period, with a mean titre of  $1/80$ . This titre is again higher than that observed by Mansfield & Kreier (1972a) in the sera of T. congolense infected rabbits, in which the response to heart antigen was greater than to kidney antigen.

The development of anti-fibrinogen antibodies was followed in rabbits 4 - 6. The average titre rose from  $1/20$  at the start of the infection to  $1/80$  at day 21.

No reduction in any of the anti-tissue activities occurred when the sera of rabbit 3 were absorbed with trypanosome antigens prior to complement fixation tests. From this it is clear that the effective antigens are true autoantigens and do not cross-react with trypanosome antigens.

TABLE 10

Reciprocal anti-heart complement fixation titres of sera  
and 19S fractions from two rabbits infected with T.equiperdum

	Days after infection	Reciprocal antibody titres <sup>a</sup>	
		Serum	19S fraction
Rabbit 2	0	20	
	3	20	
	6	20	
	11	20	20
	13	20	20
	17	20	20
	21	40	40
	24	80	20
	27	160	20
Rabbit 3	0	20	
	3	20	
	6	20	
	11	40	
	13	80	
	17	80	
	21	80	
	24	80	
	27	40	

a Values are from duplicate determinations



H. IMMUNIZATION OF RABBITS WITH ACTINOMYCIN D  
INACTIVATED TRYPANOSOMES.

Initial observations by Klein et al (1970) on the production of rheumatoid factors in rabbits infected with T. equiperdum led to the suggestion that infected rabbits might form the basis of a model for investigating the nature of rheumatoid factor formation. Another possibility was also investigated, namely that rabbits immunized with inactivated T. equiperdum would also show increased IgM levels and would provide a more successful longterm model than infected rabbits. A preliminary series of experiments was therefore carried out in an attempt to immunize rabbits against T. equiperdum.

Purified trypanosomes were inactivated at a concentration of  $5 \times 10^7$  trypanosomes/ml/200  $\mu$ g Actinomycin D, for 45 minutes at  $37^\circ\text{C}$ . In the first experiment two rabbits were immunized with  $5 \times 10^6$  trypanosomes i.e. 0.1ml of a  $5 \times 10^7$  trypanosomes/ml suspension, once per week for three consecutive weeks. On the fourth week, a challenge injection of  $1 \times 10^3$  viable trypanosomes was given i.e. 0.1ml of a  $1 \times 10^4$  trypanosome/ml suspension. Both rabbits, however, developed trypanosomiasis and died within a month of the challenge injection. (See Table 11).

Control mice were injected with each trypanosome batch prior to inactivation, and with the final challenge dose. In each case the mice died 3 - 4 days after the injection.

TABLE 11

Effect of immunizing rabbits with varying numbers of  
Actinomycin D inactivated trypanosomes.

Expt. No.	No. of Rabbits.	No. of trypanosomes in each immunization injection	Frequency of Immunization	Challenge injection No. of trypanosomes	Result
1	2	$5 \times 10^6$	1/week x 3	$1 \times 10^3$	Died
2	2	$1 \times 10^7$	1/week x 3	$1 \times 10^3$	Lived
3	2	$1 \times 10^7$	1/week x 3	$1 \times 10^3$	Lived
4a	2	$1 \times 10^7$	1/week x 3	$1 \times 10^4$	Died
4b	2	$1 \times 10^8$	1/week x 3	$1 \times 10^4$	Died

In the subsequent experiment two rabbits were immunized with  $1 \times 10^7$  inactivated trypanosomes / rabbit, injected once per week for three weeks. The trypanosomes were inactivated, as before, at a concentration of  $5 \times 10^7$  trypanosomes/ml/200  $\mu$ g Actinomycin D. A challenge injection of  $1 \times 10^3$  live trypanosomes was given in the week after the final immunization injection, and rabbits were observed for two months for the development of the disease. No symptoms of the disease were manifested in either of the two rabbits and after four months the rabbits were killed with Euthabarb (1ml/3 lbs body weight) (Goldfields Veterinary Medical Supplies, Cape Town.)

A control rabbit was injected with the challenge dose, without prior immunization, and died within  $3\frac{1}{2}$  weeks of the injection.

In a repetition of this experiment using 2 experimental rabbits and 1 control rabbit, identical results were obtained.

In order to determine the extent of the protection and the effect of an increased challenge dose, a final experiment was carried out in which 2 rabbits were immunized with  $1 \times 10^7$  Actinomycin D inactivated trypanosomes once per week for three consecutive weeks. A further 2 rabbits were immunized with  $1 \times 10^8$  inactivated trypanosomes once per week for 3 weeks. In both cases the trypanosomes were inactivated at a concentration of  $5 \times 10^7$ /ml/200  $\mu$ g Actinomycin D.

On the week following the final immunization injection, all 4 rabbits were injected with a challenge dose of  $1 \times 10^4$  live trypanosomes, and were observed for the development of disease symptoms. A control, unimmunized, rabbit was injected with the challenge dose of  $1 \times 10^4$  trypanosomes. The 4 experimental rabbits and the control rabbit all developed trypanosomiasis and died within 4 weeks of the challenge injection.

Unfortunately rheumatoid factors were not detected in the sera of infected rabbits, with the result that rheumatoid factor tests were not carried out in the immunized rabbits.

## V DISCUSSION AND CONCLUSIONS.

### A PURIFICATION OF IgM AND THE PRODUCTION OF ANTISERA.

The IgM macroglobulins are commonly isolated from serum by an initial selective precipitation, followed by fractionations based primarily on molecular size or on a combination of size and charge. The usual contaminant of IgM is  $\alpha_2$  macroglobulin, which is similar to IgM in molecular size (820,000 daltons) and in solubility in neutral salt solutions, but has a pI of 5.4 as opposed to that of IgM which is 6.1. Methods for separation of IgM from the  $\alpha_2$  macroglobulin in the fraction obtained from gel filtration are therefore as a rule based on the difference in surface charge eg by zone electrophoresis on starch, Pevikon, or agar (Onoue et al 1967). In the present studies, zone electrophoresis was performed in a sucrose gradient using a method developed by Polson & Cramer (1958) for the electrophoresis of viruses. This zone electrophoresis technique was found to have two disadvantages: one was that the concentration of protein placed on the column was limited to 10mg (total volume of 2ml): the second was the IgM denaturation, which occurred as a result of the long time taken by the electrophoresis. Zone electrophoresis in a sucrose gradient, was initially performed at pH 6.1, in order to reduce the migration of the IgM relative to that of  $\alpha_2$  macroglobulin, but extensive IgM denaturation occurred. Subsequent zone electrophoresis was therefore conducted at pH 8.6, with less denaturation of IgM. Incomplete separation of IgM

and.../

and  $\alpha_2$  macroglobulin was however, achieved after three days electrophoresis at pH 8.6. Anti  $\alpha_2$  macroglobulin antibodies were present in the antiserum produced by the immunization of a goat with the zone electrophoresis fraction.

Since a wide range of IgM mobilities have been reported (Kunkel 1960), a broad IgM band could be expected in the electrophoresis column. However, in the present experiments, only the IgM fractions from the middle of the protein peak were pooled and tested, which should have enhanced purification. (See Fig.9). Since the stated electrophoretic mobility of  $\alpha_2$  macroglobulin at pH 8.6 is  $4.2 \times 10^{-5} \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$ , and that of IgM at pH 8.6 is  $2.1 \times 10^{-5} \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$  (Schultze and Heremans 1966), these results could be explained if  $\alpha_2$  macroglobulin also has a wide range of mobilities, such that a minute concentration of  $\alpha_2$  macroglobulin has a similar mobility to that of IgM and bands in the same position. However, by immunoelectrophoretic analysis of the rabbit IgM purified by zone electrophoresis, no  $\alpha_2$  macroglobulin nor  $\beta$  lipoprotein were detected, although since both  $\alpha_2$  macroglobulin and  $\beta$  lipoprotein are extremely immunogenic (Cambier and Butler 1974) even a minute trace could have been sufficient to stimulate antibody production.

Incomplete separation of IgM and  $\alpha_2$  macroglobulin after gel filtration and electrophoresis in a Sephadex G100 column has also been reported by Van Dalen et al (1967). The removal of rabbit  $\alpha_2$  macroglobulin from rabbit IgM preparations is

reported to be .../

reported to be particularly difficult, and separation methods based on selective precipitation with zinc sulphate, which were used successfully on cow, pig and human sera were inadequate for the purification of rabbit IgM (Cambier and Butler 1974).

Another frequent contaminant of IgM is  $\beta$  lipoprotein which has a molecular weight of 3,200,000, and a pI of 5.4 (Schultze and Heremans 1966). Removal of  $\beta$  lipoprotein from IgM preparations is usually by means of either ultracentrifugation, or precipitation with dextran sulphate.

In the present studies the first step in the preparation of rabbit IgM from the serum of rabbits infected with T. equiperdum was the removal of  $\beta$  lipoproteins by centrifugation at 0°C. It was, however, noted that the goat antiserum produced after immunization with IgM contained anti  $\beta$  lipoprotein antibodies implying that traces of  $\beta$  lipoprotein remained in the IgM preparation. An additional centrifugation of dextran sulphate precipitation step should have been included in the IgM preparation procedure, although Van Dalen et al (1967) detected traces of  $\beta$  lipoprotein in the IgM preparation, even after two centrifugation steps and a  $\beta$  lipoprotein precipitation step with dextran sulphate and calcium chloride. It is therefore exceedingly difficult to completely remove  $\beta$  lipoprotein from serum, using this method so that absorption of an IgM antiserum with  $\beta$  lipoprotein is probably the most suitable alternative.

In view of the inadequate purification of IgM by euglobulin precipitation, gel filtration and zone electrophoresis, alternative methods were tried. By precipitating rabbit serum with 4% PEG at pH 7, it was hoped that only  $\gamma$  globulins would be precipitated (Polson et al 1964).  $\gamma$  globulins begin to be precipitated with addition of 2% PEG and with 6% PEG, IgM is completely absent from the supernatant.  $\alpha_2$  Macroglobulin, on the other hand, could no longer be detected in the supernatant, at a concentration of 10 - 12% PEG. (Chesbro<sup>E</sup><sub>A</sub> and Svehag 1968).  $\beta$  lipoproteins and minute traces of  $\alpha_2$  macroglobulin may, however, precipitate at 4% PEG and such traces would have to be removed by electrophoretic means. Polson et al (1964), however<sup>W</sup><sub>A</sub>, found no  $\alpha_2$  macroglobulin precipitation at PEG concentrations less than 10%, but considerable  $\beta$  component precipitation at PEG concentrations between 4 and 14%. (See Fig. 39).

The purity of the rabbit IgM prepared in this study by 4% PEG precipitation and gel filtration on Sephadex G200, was determined by immunoelectrophoresis, but this method could have left minute traces of both  $\beta$  lipoprotein and  $\alpha_2$  macroglobulin undetected. The more sensitive analytical method, i.e. that of immunizing animals with the preparation was not carried out. It is therefore, not possible to decide whether these preparations were of superior quality to those purified by zone electrophoresis.



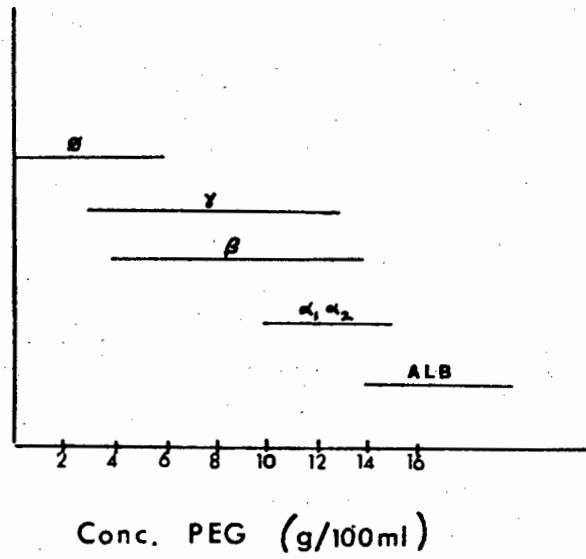


Fig. 39. PEG concentration ranges, where the different plasma components are precipitated at pH 7.0  $\phi$ , fibrinogen;  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ ,  $\gamma$ , the respective globulins; Alb, albumin.

(Polson et al, 1964).

Since commercially prepared goat anti rabbit IgM serum was not readily available, the antiserum was prepared in the laboratory. However, because of the contamination of the rabbit IgM a series of absorption experiments were conducted on the antiserum. Attempts to remove anti  $\alpha_2$  macroglobulin and anti  $\beta$  lipoprotein antibodies with rabbit neonatal serum were unsuccessful as IgM was present in the serum. This was surprising since there is complete absence of maternofetal IgM transfer in humans and in many animals (Gitlin and Gitlin 1975). It was thought that little or no maternofetal IgM transfer would occur in the rabbit, and that endogenous foetal IgM production would be minimal. The observations presented here, have recently been confirmed by Shek and Dubiski (1975), who reported maternofetal IgM transfer, through yolk sac splanchnopleur in the rabbit.

Further attempts to absorb the goat antiserum with  $\alpha_2$  macroglobulin and  $\beta$  lipoprotein failed owing to incomplete separation of IgM and  $\alpha_2$  macroglobulin.

The development of anti  $\alpha_2$  macroglobulin antibodies during the immunization of sheep or goats with highly purified rabbit IgM, was also reported by Klein et al (1970), and by Seed et al (1969). Absorptions were performed with purified  $\alpha_2$  macroglobulin (Klein et al 1970) or with a commercial preparation which is now no longer obtainable, (Seed et al 1969).

As non-specific antiserum resulted from immunization with 19S IgM it was suggested that purified monomeric or oligomeric IgM fragments might be more successful for purposes of antiserum production. Their separation from  $\alpha_2$  macroglobulin could be performed on the basis of size. In the present studies both cysteine and mercaptoethanol IgM reduction resulted in heterogenous products, the separation of which was unsuccessful. The production of IgM reduction intermediates has frequently been reported, particularly in the case of mercaptoethanol reduction (Miller & Metzger 1965, Chesbro<sup>e</sup> & Svehag 1969, and Frank & Humphrey 1969). IgM reduction was, however, successfully carried out with 2mM dithiothreitol (DTT) and although both monomeric and oligomeric IgM forms were produced, separation was obtained by density gradient centrifugation.

Parkhouse (1975) reported the re-aggregation of the IgM subunits (IgM<sub>S</sub>) and HL subunits formed after partial reduction with DTT, through the formation of non-covalent bonds. When an oligomeric structure was present, non-covalent forces between it and IgM subunits caused the formation of 19S IgM or even larger aggregates.

It is well known that the last bonds to be formed in the synthesis of IgM are the first to be reduced, and are those bonds between the individual IgM monomers. (Bevan et al 1972). On the basis of this it is thought that incomplete IgM reduction with mercaptoethanol and cysteine resulted in the presence of both oligomers and monomers, from which 19S IgM could have been formed by non-covalent bonds (See Fig. 16).

Because of the results obtained by DTT reduction of rabbit serum, in which  $\alpha_2$  macroglobulin was apparently reduced and the inability to locate the reduction products, monomeric IgM was not used for the production of anti IgM serum. Traces of  $\alpha_2$  macroglobulin might have contaminated the 7S fraction. The disappearance of  $\alpha_2$  macroglobulin in serum, does not necessarily imply destruction of possible minute amounts contaminating the "purified" IgM. Since  $\alpha_2$  macroglobulin consists of eight polypeptide chains (Putnam 1975), which on reduction form dimers, there is a strong possibility that reduction of the molecule would occur. Nielsen (1976) however, reported no reduction of  $\alpha_2$  macroglobulin with 0.2M mercaptoethanol. He produced a  $\mu$  chain specific antiserum by immunizing animals with monomeric IgM, and subsequently absorbing the antiserum with glutaraldehyde-polymerized 7S serum proteins obtained from gel filtration.

Future attempts to prepare a nonspecific anti IgM serum should include immunization with both 7S and 13.4S IgM preparations obtained by mild DTT reduction, since this treatment may not reduce  $\alpha_2$  macroglobulin. Alternatively semi-purified IgM could be treated with 5M urea, in order to dissociate  $\alpha_2$  macroglobulin to subunits with sedimentation rates extending down to 9S, which could then be removed by gel filtration (Schultze & Heremans 1966). Since 5M urea can dissociate  $\alpha_2$  macroglobulin into smaller subunits, a fact which was unfortunately not realised at the time when serum IgM concentration determinations were being carried out, accurate 19S IgM concentrations could have been determined by analytical ultracentrifugation.

A third possibility could be considered, namely that of gel filtration of partially pure IgM on a Biogel column of 3 metres on which  $\alpha_2$  macroglobulin can be separated from IgM (Chesbro & Svehag 1969).

In summary IgM was purified by a combination of euglobulin precipitation, gel filtration and zone electrophoresis. The goat antiserum produced after immunization with this IgM, reacted not only with IgM but also with  $\alpha_2$  macroglobulin and with  $\beta$  lipoprotein. Absorption of these antibodies from the antiserum was unsuccessful. Further preliminary attempts were made to purify IgM by PEG precipitation, but low IgM yields prevented further antiserum production. Attempts to purify monomeric IgM, after 19S IgM reduction were successful, but goat immunization with this IgM was not tried.

#### B. IMMUNOGLOBULIN LEVELS.

The results of preliminary studies by quantitative immunoelectrophoresis on the IgM concentrations in the sera from infected rabbits supported the findings of Klein et al (1970) that the IgM concentrations in T. equiperdum infected rabbits are greatly increased at the peak of the infection. However, as there are inherent inaccuracies in quantitative immunoelectrophoresis, and as it is difficult to detect the endpoint, these preliminary results were considered as no more than a general indication of macroglobulinaemia.

possible that storage conditions used in the present studies were more prolonged and extreme than those cited above.

The IgM levels determined in the freshly collected rabbit sera by both rocket immunoelectrophoresis and gel diffusion were of a similar order of magnitude to those reported in previous studies of various trypanosome infections, (Mattern et al 1963, Masseyeff & Lamy 1966, Houba et al 1969, Capbern et al 1974, and Clarkson et al 1975). Frommel et al (1970) using radial immunodiffusion tests showed in rabbits infected with T. equiperdum an elevenfold increase of IgM at day 21 over the normal level. In the present studies (Table 5) an approximate tenfold increase was found.

Klein et al (1970) determined the concentration of IgM in rabbits infected with T. equiperdum by an indirect method; i.e. the  $\alpha_2$  macroglobulin content, measured by radial immunodiffusion, was subtracted from the total macroglobulin content, derived from 19S ultracentrifuge diagrams. The 19S IgM increased from an average normal level of 1.3mg/ml to a maximum concentration of 7.9mg/ml after 20 days, which corresponded to a sixfold increase. These results agree with those in Fig 26 despite the fact that the concentration of  $\alpha_2$  macroglobulin was not subtracted from the total 19S content.

Attempts to measure the  $\alpha_2$  macroglobulin concentration in the Model E after IgM reduction, in order to obtain accurate 19S IgM determinations, were unsuccessful, owing to the denaturation of  $\alpha_2$  macroglobulin. Further studies on the

quantitation ...../

quantitation of  $\alpha_2$  macroglobulin and 19S IgM should be conducted by selectively dissociating the  $\alpha_2$  macroglobulin with 5M urea.

Although the presence of IgM<sub>N</sub> in the sera from infected patients and animals has been reported previously, no satisfactory concentration determinations have been published. Frommel et al (1970), using a semi-quantitative estimation, with artificially produced monomers as a standard, found that 7S IgM was less than 5% of the total IgM level. Considerable difficulty is experienced in measuring IgM<sub>N</sub> since quantitation after the separation of 19S and 7S IgM (IgM<sub>N</sub>) results in reduced concentrations owing to inevitable IgM denaturation. The alternative electrophoretic technique of quantitation are not easily standardized since the artificially produced IgM monomers (IgM<sub>S</sub>) used as reference proteins, are more negatively charged than pentameric IgM, but less negatively charged than naturally occurring IgM<sub>N</sub>. (Hansson & Laurell 1970). In techniques such as rocket immunoelectrophoresis, where peak height is proportional to antigen concentration, the difference in electrophoretic mobility between IgM<sub>S</sub> and IgM<sub>N</sub> could cause considerable error, and for this reason no accurate 7S IgM determinations were attempted in this study.

The concentrations of IgG in infected rabbits have been reported to rise from an average of 13mg/ml to a maximum of 39mg/ml (Klein et al 1970). Frommel et al (1970) however, reported a somewhat lower preinfection concentration of 6.6mg/ml which is in close agreement with that obtained in this work. IgG levels determined by double innumodiffusion (Fig. 26) rose from an average 6.7 mg/ml before infection to a maximum of 50 mg/ml.

It is interesting to note that Klein et al (1970) reported that IgG levels rose 5 - 10 days after the IgM increase, whereas in Fig. 26 both IgG and IgM increase almost simultaneously.

In conclusion a tenfold increase in the level of 19S IgM in the serum from trypanosome infected rabbits was found, by gel diffusion. Attempts to measure the 7S IgM concentrations accurately were unsuccessful, owing to the difficulty in standardizing rocket immunoelectrophoresis. IgG concentrations in infected rabbits were found to rise simultaneously with the IgM to a maximum of 50mg/ml corresponding to a  $7\frac{1}{2}$  fold increase.

#### C. THE ABSENCE OF RHEUMATOID FACTORS (RFs)

The presence of rheumatoid factors in sera from patients with trypanosomiasis caused by T. gambiense and T. rhodesiense has been described by Klein and Mattern (1965) and by Houba and Allison (1966), the frequency of the detection of significant levels of RFs varying from 2 - 4.5% by the latex fixation test. As outlined in Chapter II, rheumatoid factors have also been detected in rabbits infected with T. equiperdum (Klein et al 1970) but not in mice infected with T. gambiense, T. rhodesiense or T. brucei. Houba et al (1969) also failed to detect rheumatoid factors in the sera of trypanosome infected monkeys.



TABLE 12.

Latex and Waaler-Rose titres in sera from rabbits  
infected with T. equiperdum (Klein et al 1970)

Rabbit No. <sup>a</sup>	Days after infection.	Reciprocal latex titres.	Reciprocal Waaler-Rose titres.	Reciprocal Waaler-Rose titres unsensitized
Rabbit 13				
( 3	0	< 7	2	< 2
out of	5	< 7	2	< 2
13	10	112	n/d	n/d
rabbits.)	20	224	64	32
	25	224	4	8
		56	< 2	< 2
Rabbit 3				
( 3	0	< 7	64	4
out of	4	< 7	32	8
13	10	28	64	8
rabbits.)	15	112	8	8
	20	112	4	4
Rabbit 2				
( 7	0	7	< 2	< 2
out of	6	28	< 2	< 2
13	12	7	< 2	< 2
rabbits.)	17	56	< 2	< 2
	22	56	< 2	< 2
	27	56	< 2	< 2

a. Rabbit numbers in brackets indicate a similarity in the overall pattern of results.

Furthermore it is interesting that although Klein et al (1970) reports the "predilection" of their rheumatoid factors for a heterologous substrate ie. human IgG in latex fixation, no comparable latex fixation studies were conducted with the homologous substrate, ie rabbit gamma globulins. In the Waaler-Rose test, however, human erythrocytes were sensitized with rabbit gamma globulin, and it was on the basis of a comparison of these results with the latex fixation results, that the conclusion of the rheumatoid factor predilection for a heterologous substrate was drawn. In view of the discrepancies frequently found between the Waaler-Rose results and the latex fixation results (Griebble et al 1969), the validity of this comparison may be questioned.

The latex fixation and passive haemagglutination results presented in this thesis do not support the claim of Klein et al (1970) that T. equiperdum infections in the rabbit consistently lead to the production of rheumatoid factors. Despite the use of a variety of sensitizing globulins and attempts to dissociate rheumatoid factor aggregates and to detect rheumatoid factors in the cryoglobulin fraction of the sera, results remained consistently negative.

The most likely explanation for the occurrence of rheumatoid factors in patients with trypanosomiasis (if indeed the frequency of rheumatoid factor occurrence is significant) but not in trypanosome infected experimental animals is the nature of the host-parasite relationship.

It may be relevant that T. gambiense and T. rhodesiense circulate freely in both the blood and cerebrospinal fluid of the patient whereas T. equiperdum is mainly a tissue parasite and its localization may be less favourable for the stimulation of rheumatoid factors.

In conclusion rheumatoid factors were not detected in T. equiperdum infected rabbits by latex fixation, nor by passive haemagglutination despite the use of a variety of sensitizing antigens. From the preceding discussion of the results of Klein et al (1970) it is clear that the significance and validity of their results are questionable.

#### D. ANTI - TRYPANOSOMAL ACTIVITY OF IMMUNOGLOBULINS.

Despite the large and persistent increase in IgM in trypanosomiasis only a small proportion of the IgM is specific for trypanosome antigens (Masseyeff 1969).

In fact the IgM fraction has also been shown to contain significant concentrations of autoimmune antibodies against rabbit tissues and heterophile antibodies (Houba & Allison 1966, Seed & Gam 1967, Houba et al 1969).

The presence of anti-trypanosome activity in the IgM serum fractions has seldom been investigated, but in all cases the titres were low compared to the extent of the macroglobulinaemia (Takayanagi & Enriquez 1973). Seed et al (1969) reported that the IgM fraction of serum from infected rabbits contained antibodies agglutinating T. gambiense, at a maximum titre of  $1/512$  on the twelfth day after infection.

In the present study, IgM anti-trypanosome antibodies were not detected by immunofluorescence (Fig.34), in rabbits infected with T. equiperdum but IgM anti-trypanosome complement fixation titres of  $1/640$  -  $1/1280$  and agglutination titres of  $1/640$  -  $1/1280$  were observed. It is interesting to note that the agglutination titres were higher than those reported by Seed et al (1969), and the complement fixation titres higher than those reported by Mattern et al (1961). The complement fixation titres are also greater than those obtained against tissue antigens (Fig. 35), so that although not all of the IgM can be accounted for by the anti-trypanosome activity, perhaps a more significant proportion of IgM is directed towards the trypanosome antigens than previously thought.

The IgG anti-trypanosome activity measured by agglutination was reported by Seed et al (1969), to reach a maximum titre of  $1/32$  at day 12. In the present work IgG anti-trypanosome titres of  $1/40$  and  $1/320$  at day 13 were determined by immunofluorescence and complement fixation respectively, whereas maximum titres of  $1/80$  and  $1/1280$  were observed at day 17. It is questionable, of course, whether a comparison between the results of Seed et al (1969) obtained with T.gambiense and the present work with T.equiperdum is truly justified, since the two organisms differ in their effect and circulation of the animal.

An alternative explanation for the discrepancies between the anti-trypanosome activities in T gambiense and T equiperdum infections is the phenomenon of antigenic variation. It is well known that there is a sequential variation of the variant antigens in trypanosome infections, so that discrepancies in the level of anti-trypanosome antibodies could result from the nature of the variant antigens used in the assays. The decrease in anti-trypanosome antibody titres observed after day 17 (by complement fixation tests with a standard antigen preparation, Fig.35), despite the persistent macroglobulinaemia may be interpreted as the result of antigenic variation. For an accurate and unambiguous comparison of the anti-trypanosome response between different infections, anti-trypanosome antibody assays should be performed with purified trypanosome variants.

Evidence on the role of antigenic variation as the cause of macroglobulinaemia in trypanosome infections is inconclusive, since only a relatively small proportion of the IgM is specific for the trypanosomal antigens. However, investigations have not been made with the purified variants, a study which is clearly needed in order to determine, finally, the role of antigenic variation in the cause of macroglobulinaemia.

In summary, anti-trypanosome antibodies were detected by immunofluorescence in the serum and 7S serum fractions of trypanosome infected rabbits. Although IgM anti-trypanosome

antibodies .... /

antibodies were only detected by agglutination and complement fixation, the titres were in excess of those previously reported. Two explanations for the discrepancy between the antibody responses have been discussed, namely;

- i) the effect of different infecting trypanosome species, and
- ii) the effect of antigenic variation.

#### E. AUTOIMMUNITY TO TISSUE ANTIGENS.

A number of recent studies have shown that autoantibodies are present in the serum of rabbits infected with T gambiense (Muschel et al 1961, Seed & Gam 1967) T rhodesiense (Muschel et al 1961), T congolense (Mansfield & Kreier 1972a) and T brucei (Boreham & Facer 1974, Mackenzie & Boreham 1974). A discussion of these studies has been outlined in Chapter II. The antibodies were shown to be of IgM nature, and to react with rabbit liver, heart, brain, spleen and kidney tissue as well as with a fibrinogen component. The rabbit is also known to possess natural autoantibodies which are thought to be responsible for the removal of debris (due to cell death) present in the circulatory system. (Kidd & Friedewald 1942, Rose & Brinckerhoff 1969).

Natural autoantibodies are present at lower titres in the sera than those observed in the diseased states.

In the present investigations IgM autoantibodies were detected against rabbit liver, heart and kidney tissues in rabbits infected with T equiperdum. The antibody response was greatest to liver antigen, with the least response to heart antigen. These <sup>results</sup> do not agree with those of Mansfield & Kreier (1972a) in which the antibody response to heart was greater than the response to kidney, in T congolense infected rabbits.

Serological cross-reaction of tissue components and trypanosomes was excluded by absorption studies, the results of which agree with those of Mackenzie & Boreham (1974). Furthermore since similar complement fixation results were observed with both diseased and normal tissues, it is clear that the effective antigens were not of trypanosomal origin. Cross-absorption studies with the various tissues were not carried out in this investigation, but results by Mackenzie & Boreham (1974) indicate the occurrence of common antigens.

The possible reasons for the induction of autoantibodies in experimental trypanosomiasis have been discussed in Chapter II. Since the tissue antigens to which the autoantibodies are directed, cross react it is quite possible that the autoantibody response is essentially of a non-specific nature, perhaps stimulated by minor tissue damage in, for example, the liver. It is well known that autoantibodies to both native collagen (Andriopoulos et al 1975), and denatured collagen (Michaeli & Fudenberg 1974) may occur in rheumatoid arthritis, a disease which shows many similarities to trypanosomiasis. In fact it appears that the antibodies arise in response to the destruction of

connective tissue.../

connective tissue in the inflamed joints, and thereafter react with collagen both denatured and native. It seems likely that the trypanosomes cause minor tissue damage after their invasion of the tissues, in the early stages of the infection. After the initial damage the antigens released may be common to many tissues. In the case of those trypanosomes invading the circulatory system eg T. gambiense it is possible that antibodies are first formed against denatured fibrinogen, and thereafter react with released tissue antigens.

However, an alternative suggestion was made by Greenwood & WHITTLE (1975) on the grounds that they detected free light chains in human trypanosomiasis, namely that, trypanosomes exert a non-specific mitogen effect. This hypothesis finds support in the large and persistent increase in IgM in trypanosome diseases, particularly in view of the fact that not all the IgM is directed against the trypanosomes. If the trypanosomes act as a mitogen, then the production of tissue autoantibodies may be explained on the basis of the stimulation of those antibody clones producing the small concentrations of natural autoantibody observed by Kidd & Friedewald (1942).

Clearly investigations into the possible mitogenic effect of trypanosomes are required in order to draw any final conclusions concerning the cause, both of the increased IgM response, and the production of autoantibodies in trypanosomiasis.



Preliminary experiments to investigate the mitogenic effect in rabbits infected with T. equiperdum were designed, but the results were inconclusive. Rabbits were injected with bovine serum albumin and after varying periods of time, injected with a lethal dose of trypanosomes. The 19S serum fractions were then monitored by complement fixation, for the production of anti-bovine serum albumin antibodies. An increase in the anti-BSA antibody titres after trypanosomal infection by comparison with uninfected rabbits injected with BSA would have indicated the likelihood of a mitogenic effect. Owing to the lack of anti-BSA titres in the rabbit sera no conclusion could be drawn on the mitogenic effect of trypanosomes.

This mitogenic effect seems to be the most suitable explanation for both the persistent increase in IgM and the production of autoantibodies in experimental trypanosomiasis.\*

In summary autoantibodies were observed in T. equiperdum infected rabbits against liver, kidney and heart tissues. The antibody responses were greatest to liver, and least to heart antigens. No serological cross reactivity between trypanosomal antigens and tissue antigens was observed. A possible explanation for both the persistent macroglobulinaemia and the production of autoantibodies is that trypanosomes exert a mitogenic effect.

\* Note added in Proof.

A recent demonstration that trypanosomal antigens have a mitogenic effect in vitro (Esuruoso 1976) confirms this idea.

## VI REFERENCES

- (1) ABU-SALIH, H.S., MURANT, A.F., DAFT, M.J. The use of antibody-sensitized latex particles to detect plant viruses. *J. Gen. Virol.* 1968, 3, 299-302.
- (2) ALLISON, A.C., DENMAN, A.M., BARNES, R.D., Co-operating and controlling functions of thymus-derived lymphocytes in relation to auto immunity. *Lancet*, 1971, (ii), 135-140.
- (3) ALLSOPP, B.A., NJOGU, A.R., HUMPHRIES, K.C., Nature and location of Trypanosoma brucei subgroup exoantigen and its relationship to 45 antigen. *Exp. Parasitol*, 1971, 29, 271-284.
- (4) ANDRIOPOULOS, N.A., MILLER, E.J., MESTECHY, J. The incidence of antibodies against native collagen in rheumatoid arthritis. *Fed. Proc.*, 1975, 34, 950.
- (5) ASHERSON, G.L. & DUMONDE, D.C. Characterization of autoantibodies produced in the rabbit by the injection of rat liver. *Brit. J. exp. Path.* 1962, 43, 12-20.
- (6) ASHERSON, G.L. & DUMONDE, D.C. Autoantibody production in rabbits. V. Comparison of autoantibody response after injection of rat and rabbit liver and brain. *Immunology*, 1964, 7, 1-8.
- (7) BANATVALA, J.E., BEST, J.M., KENNEDY, E.A., SMITH, E.E., & SPENCE, M.E., A serological method of demonstrating recent infection by rubella virus. *Brit. Med. J.* 1967, 3, 285-286.
- (8) BASSILY, S., MOHRAN, Y.Z., SAID, M.I., MOUSSA, M., ISKANDER, F., MOORO, H., Autoantibodies in schistosomal liver fibrosis. *J. Trop. Med. Hyg.* 1973, 76, 153-155.
- (9) BERNHARD, G.C., CHENG, W. & TALMAGE, D.W. The reaction of rheumatoid factor and complement with  $\gamma$ -globulin coated latex. *J. Immunol.* 1962, 88, 750-762.
- (10) BEVAN, M.J., PARKHOUSE, R.M.E., WILLIAMSON, A.R., & ASKONAS, B.A., Biosynthesis of immunoglobulins. *Progr. Biophys. Molec. Biol.* 1972, 25, 131-162.
- (11) BOREHAM, P.F.L., Immune reactions and kinin formation in chronic trypanosomiasis. *Br. J. Pharmac. Chemother.* 1968, 32, 493-504.

- (12) BOREHAM, P.F.L., & FACER, C.A., Autoimmunity in trypanosome infections - II. Anti-fibrin/fibrinogen (anti-F) autoantibody in Trypanosoma (Trypanozoon) brucei infections of the rabbit. Int. J. Parasit., 1974, 4, 601-607.
- (13) BRADSTREET, C.M.P. & TAYLOR, C.E.D., Technique of complement fixation test applicable to the diagnosis of virus disease. Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service, 1962, 21, 96-104.
- (14) BROWN, R.K. & BAKER, W.H. & PETERKOFKY, A., KAUFFMAN, D. L., The extraction of glycoproteins from fraction III of human sera. J. Am. Chem. Soc. 1954, 76, 4244-4245.
- (15) BUSH, S.T., SWEDLUND, H.A., GLEICH, G.J., Low molecular weight IgM in human sera. J. Lab. Clin. Med. 1969, 73, 194-201.
- (16) CAMBIER, J.C. & BUTLER, J.E. A rapid method for the purification of immunoglobulin M (IgM) from the sera of certain mammalian species. Prep. Biochem., 1974, 4, 31-46.
- (17) CAMPBELL, D.H., GARVEY, J.S., CREMER, N.E. & SUSSDORF, D.H. Methods in Immunology, 2nd Ed. W.A. Benjamin Inc. New York, 1970.
- (18) CANTRELL, W. Mutation rate and antigenic variation in Trypanosoma equiperdum. J. Infect. Dis. <sup>1958</sup> 103, 263-271.
- (19) CAPBERN, A., MATTERN, P., & PAUTRIZEL, R. Etude comparative du taux <sup>des</sup> protéines sériques au cours de trypanosomoses à Trypanosoma Gambiense et à Trypanosoma Cruzi chez la souris. Exp. Parasitol, 1974, 35, 86-91.
- (20) CECIL, R.L., NICHOLS, E.E., & STAINSBY, W.J., The etiology of rheumatoid arthritis. Amer. J. Med. Sci., 1931. 181. 12-25.
- (21) CHENG, C.T. & PERSELLIN, R.H. Interference by Clq in slide latex tests for rheumatoid factor. Ann. Intern Med., 1971, 75, 683-686.

- (22) CHENG, C.T. & PERSELLIN, R.H., Studies with rabbit IgG-coated latex for the detection of rheumatoid factor. J. Med., 1973, 4, 88-95.
- (23) CHESEBRO, B. & SVEHAG, S.E. Precipitation of human serum proteins by polyethyleneglycol. Clin. Chim. Acta., 1968, 20, 527-529.
- (24) CHESEBRO, B. & SVEHAG, S.E. Biological activity and morphology of three types of rabbit IgM antibody subunits. J. Immunol. 1969, 102, 1064-1073.
- (25) CLARKSON, M.J., PENHALE, W.J., & MCKENNA, R.B., Progressive serum protein changes in experimental infections of calves with Trypanosoma vivax. J. comp. Path. 1975, 85, 397-410.
- (26) CROSS, G.A.M., Identification and purification of a class of soluble surface proteins from Trypanosoma brucei. Trans. R. Soc. Trop. Med. Hyg., 1973, 67, 261.
- (27) CROSS, G.A.M., Identification, purification, and properties of clone-specific glyco-protein antigens constituting the surface coat of Trypanosoma brucei. Parasitology. 1975, 71, 393-417.
- (28) CROWLE, A.J., Immunodiffusion. Second Edition, Academic Press. New York. 1973.
- (29) CSIZMAS, L., Preparation of formalinized erythrocytes. Proc. Soc. exp. Biol. Med. 1960, 103, 157-160.
- (30) CUNNINGHAM, M.P. & VICKERMAN, K., Antigenic analysis in the Trypanosoma brucei group, using the agglutination reaction. Trans. R. Soc. Trop. Med. Hyg., 1962, 56, 48-59.
- (31) DAMMACCO, F., GIUSTINO, V., & BONOMA, L. The occurrence of serum 7S IgM in disease associated with immunoglobulin abnormalities. Int. Arch. Allerg., 1970, 38, 618-626.
- (32) DANIEL, T.M. & STAVITSKY, A.B., Passive haemagglutination in study of antigens and antibodies in "Methods in Medical Research" (H. Eisen), 1964, 10, 152-157.

- (33) DAVIS, B.D. Biologic false positive serological tests for syphilis. *Medicine (Baltimore)* 1944, 23, 359-414.
- (34) DESOWITZ, R.S. In "Immunity to Parasitic Animals" (G.J. Jackson, R. Herman, I. Singer) Vol 2. 1970. 551-596. Appleton-Century-Crofts, New York.
- (35) DEUTSCH, H.F. & MORTON, J.I. Human Serum macroglobulins and dissociation units. I. Physiochemical properties. *J. Biol. Chem.*, 1958, 231, 1107-1118.
- (36) DIEHL, E.J., RISBY, E.L. Serum changes in rabbits experimentally infected with Trypanosoma gambiense. *Am J. Trop. Med. Hyg.* 1974. 23, 1019-1022.
- (37) DUXBURY, R.E. ANDERSON, J.S., WELLDE, B.T., SADUN, E.H., & MURIITHI, I.E. Trypanosoma congolense: Immunization of mice, dogs and cattle with gamma-irradiated parasites. *Exp. Parasitol.* 1972, 32, 527-533.
- (38) DUXBURY, R.E. & SADUN, E.H. Resistance produced in mice and rats by inoculation with irradiated Trypanosoma rhodesiense. *J. Parasitol.* 1969, 55, 859-865.
- (39) EDELMAN, G.M., KUNKEL, H.G. & FRANKLIN, E.C., Interaction of rheumatoid factor with antigen - antibody complexes and aggregated gamma globulin. *J. exp. Med.*, 1958, 108 105-120.
- (40) ESKELAND, T. & HARBOE, M. 7S Immunoglobulin M (IgM) from serum purified by anti- $\mu$ - chain immunoabsorbent: Comparison of the Reassociation properties of 7S IgM and various 19 S IgM subunits. *Scand. J. Immunol.* 1973, 2, 511-522.
- (41) ESURUOSO, G.O., The demonstration in vitro of the mitogenic effects of trypanosomal antigen on the spleen cells of normal, athymic and cyclophosphamide treated mice. *Clin. exp. Immunol.*, 1976, 23, 314-317.
- (42) FAHEY, J.L. & McKELVEY, E.M., Quantitative determination of serum immunoglobulins in antibody-agar plates. *J. Immunol.* 1965, 94, 84-90.

- (43) FERNANDES, J.F., HALSMAN, M., & CASTELLANI, O.  
Effect of actinomycin D on the infectivity of Trypanosoma cruzi. Nature (London), 1965, 207, 1004-1005.
- (44) FRANK, M.M. & HUMPHREY, J.H., Spontaneous re-aggregation of IgM subunits & restoration of antibody activity after reduction & alkylation of rabbit anti-Forssman antibody. Immunology, 1969, 17, 237-247.
- (45) FRANKLIN, E.C. HOLMAN, H.R. MÜLLER-EBERHARD, H.J. & KUNKEL, H.G., An unusual protein component of high molecular weight in the serum of certain patients with rheumatoid arthritis. J. exp. Med. 1957, 105, 425-438.
- (46) FROMMEL, D., PEREY, D.Y.E., MASSEYEFF, R., & GOOD, R.A., Low molecular weight serum immunoglobulin M in experimental trypanosomiasis. Nature (London), 1970, 228, 1208-1210.
- (47) GAARDER, P.I., NATVIG, J.B. Hidden rheumatoid factors reacting with "non a" and other antigens of native autologous IgG. J. Immunol., 1970, 105, 928-937.
- (48) GELMAN Handbook on Clinical Electrophoresis. 1970. Gelman Instrument Company, Ann Arbor. Michigan.
- (49) GITLIN, D., & GITLIN, J., Fetal and neonatal development of human plasma proteins. in "Plasma Proteins Vol II" (F.W. Putman), 1975. 263-319. Academic Press, New York.
- (50) GLEICH, G.J. UHR, J.W., VAUGHAN, J.H. & SWEDLUND, H.A., Antibody formation in dysgammaglobulinaemia. J. Clin. Invest., 1966, 45, 1334-1340.
- (51) GOIDL, E.A., & MARSDEN, P.D., Immunoglobulins in African trypanosomiasis. Lancet, 1974, I, 690.
- (52) GOLDMAN, M. Fluorescent antibody methods. 1968. Academic Press, New York.
- (53) GOODWIN, L.G. The pathology of african trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg. 1970, 64, 797-812.

- (54) GOODWIN, L.G. The African scene: mechanisms of pathogenesis in trypanosomiasis, in "Trypanosomiasis & Leishmaniasis with special reference to Chagas' disease." 1974. 107-124. Ciba Foundation symposium 20 (new series) Elsevier. Excerpta Medica. North Holland.
- (55) GOODWIN, L.G., GREEN, D.G. GUY, M.W. & VOLLER, A., Immunosuppression during trypanosomiasis. Br. J. exp. Pathol. 1972, 53, 40-43.
- (56) GOODWIN, L.G., GUY, M.W. & BROOKER, B.E., Connective tissue changes in rabbits infected with Trypanosoma (Trypanozoon) brucei. Parasitology. 1973, 67, 115-122.
- (57) GRAY, A.R., The influence of antibody on serological variation in Trypanosoma brucei. Ann Trop. Med. Parasitol., 1962, 56, 4-13.
- (58) GRAY, A.R., Antigenic variation in a strain of Trypanosoma brucei transmitted by Glossa morsitans and G. palpalis. J. Gen. Microbiol. 1965, 41, 195-214.
- (59) GREENWOOD, B.M., Possible role of a B-cell mitogen in hypergammaglobulinaemia in malaria and trypanosomiasis. Lancet, 1974, i, 435-436.
- (60) GREENWOOD, B.M. & WHITTLE, H.C., Production of free light chains in gambian trypanosomiasis. Clin. exp. Immunol. 1975, 20, 437-442.
- (61) GRIEBEL, H.G., BACH, G.L. SZANTO, M., & ANDERSON, T.O. The serologic diagnosis of rheumatoid arthritis. The latex-rabbit gamma-globulin fixation test. J. chron. Dis. 1969, 21, 667-676.
- (62) HANSSON, U.B., & LAURELL, C.B., Electrophoretic and immunologic difference between monoclonal IgM molecules and their subunits in native plasma. Immunochemistry, 1970, 7, 559-565.
- (63) HARBOE, H. RAV, B., & AHO, K., Properties of various anti-globulin factors in human sera. J. exp. Med. 1965, 121, 503-519.
- (64) HERBERT, W.J. LUMSDEN, W.R. & FRENCH, A.M., Survival of trypanosomes after rapid cooling and storage at -196°C. Trans R. Soc. Trop. Med. Hyg. 1968, 62, 209-212

- (65) HERBERT, W.J., MUCKLOW, M.G. & LENNOX, B., The cause of death in acute murine trypanosomiasis. Trans R. Soc. Trop, Med. Hyg., 1975, 69, 4.
- (66) HERBERT, W.J. & WILKINSON, P.C. A Dictionary of Immunology. 1971. Blackwell. Oxford.
- (67) HOARE, C.A. The Trypanosomes of Mammals. Blackwell Scientific Publications. Oxford. 1972.
- (68) HOLLEY, H.L. HAMMACK, W.J. & DOUGLAS, C. The application of the sensitized sheep cell test in sera of patients with non-rheumatic diseases and false positive serological reactions for rheumatoid arthritis. Am.J. Med. Sci. 1961. 242, 331-337.
- (69) HORESJI, J & SMETANA, R., The isolation of gamma globulin from blood-serum by Rivanol. Acta Med. Scand. 1956, 155, 65-70.
- (70) HOUBA, V & ALLISON, A.C. M-antiglobulins (Rheumatoid-factor-like globulins) and other gamma globulins in relation to tropical parasitic infections. Lancet, 1966, i, 848-852.
- (71) HOUBA, V., BROWN, K.N. & ALLISON, A.C., Heterophile antibodies and <sup>M-antiglobulins</sup> immunoglobulins in experimental trypanosomiasis. Clin. exp. Immunol, 1969, 4, 113-123.
- (72) HOUBA, V., & LAMBERT, P.H. , A new technique for the separation of high and low molecular components of immunoglobulins M. J. Immunol. Methods, 1974. 5, 353-358.
- (73) HUGHES, T.P. A precipitin reaction in yellow fever. J. Immunol., 1933, 25, 275-294.
- (74) JONES, D. Low molecular weight IgM as a pitfall in immunoglobulin quantitation by radial diffusion. Clin. Chim. Acta, 1970, 29, 551-556.
- (75) KIDBY, R. Immunofluorescent detection and quantitation of autoantibodies - development of a routine service. Med. Lab. Tech. 1974, 31, 305-310.



- (76) KIDD, J.G. & FRIEDEWALD, W.F., A natural antibody that reacts in vitro with a sedimentable constituent of normal tissue cells. II Specificity of the phenomenon = general discussion. J. exp. Med., 1942, 76, 557-578.
- (77) KIMBER, C.D., A method for diagnosing human trypanosomiasis using the indirect fluorescent antibody technique. Rep. E. Afr. Trypan. Res. Org. 1966, 1965, 31-32.
- (78) KLEIN, F. & MATTERN, P., Rheumatoid factors in primary and reactive macroglobulinaemia. Ann. Rheum. Dis. 1965, 24, 458-464.
- (79) KLEIN, F., MATTERN, P., RADEMA, H., & VAN ZWET, T.L. Slowly sedimenting serum components reacting with anti-IgM sera. Immunology. 1967, 13, 641-647.
- (80) KLEIN, F., MATTERN, P., KORNMAN, V.D., & BOSCH, H.J. Experimental induction of rheumatoid factor-like substances in animal trypanosomiasis. Clin. exp. Immunol. 1970, 7, 851-863.
- (81) KLEIN, F., MATTERN, P., & MEUWISSEN, J.H.E.T., Experimental anti-γ-G-globulins in infectious diseases, in "Human anti-human gammoglobulins" (R. Grubb, G. Samuelsson,) P.63-69., 1971. Pergamon Press. Oxford and New York.
- (82) KUNKEL, H.G. Macroglobulins & high molecular weight antibodies. in "The Plasma Proteins", 1960. Vol I. (F.W. Putnam, ed.), p. 279-307. Academic Press. New York.
- (83) LANHAM, S.M., Separation of trypanosomes from the blood of infected rats and mice by anion exchangers. Nature, (London), 1968, 218, 1273-1274.
- (84) LINDSTRÖM, F.D. Hidden rheumatoid-factor in active seronegative rheumatoid arthritis. Scand. J. Rheumatology, 1973, 2, 133-135.
- (85) LOSOS, G.J., PARIS, J., WILSON, A.J. & DAR, F.K. Distribution of Trypanosoma congolense in tissues of cattle. Trans. R. Soc. Trop. Med. Hyg. 1973, 67, 278.

- (86) LOSPALLUTO, J. Low molecular weight IgM in sera of patients with rheumatoid arthritis. *Arthritis. Rheum.*, 1968, II, 831 only.
- (86) LUMSDEN, W.H.R., HERBERT, W.J. & McNEILLAGE, G.J.C., *Techniques with Trypanosomes*. Churchill Livingstone, Edinburgh, 1973.
- (87) LYET, J.P. & NORMANSELL, D.E., Low molecular weight rheumatoid factors in rheumatoid arthritis sera. *Immunochemistry*, 1974, II, 417-421.
- (88) MacKENZIE, A.R., & BOREHAM, P.F.L., Autoimmunity in Trypanosome Infections. I. Tissue autoantibodies in Trypanosoma (Trypanozoon) brucei infections of the rabbit. *Immunology*, 1974, 26, 1225-1238.
- (89) MANSFIELD, J.M. & KREIER, J.P., Autoimmunity in experimental Trypanosoma congolense infections of rabbits. *Infect. Immun.* 1972a, 5, 648-656.
- (90) MANSFIELD, J.M. , & KREIER, J.P. Tests for antibody and cell-mediated hypersensitivity to trypanosome antigens in rabbits infected with Trypanosoma congolense. *Infect. Immun.* 1972b, 6, 62-67.
- (91) MANSFIELD, J.M. & WALLACE, J.H. Suppression of cell-mediated immunity in experimental African trypanosomiasis. *Infect. Immun.*, 1974, 10, 335-339.
- (92) MASSEYEFF, R., Les immunoglobulines au cours des trypanosomiasés. In: "Synthèse cellulaire et structure moléculaire des immunoglobulines." (B. Halpern), 1969, p. 339-367, Dunod, Paris.
- (93) MASSEYEFF, R., BLONDEL, J., & MATTERN P., Low molecular weight IgM in the serum and cerebrospinal fluid of patient infected with Trypanosoma gambiense. *Z. Immun.-Forsch.* 1972, 143, 291-303.
- (94) MASSEYEFF, R., & LAMY, J., Taux des immunoglobulines sériques au cours de la trypanosomiase africaine à Trypanosoma gambiense. *Clin. Chim. Acta.* 1966, 14, 285-292.
- (95) MATTERN, P. Techniques et intérêt épidémiologique du diagnostic de la trypanosomiase humaine africaine par la recherche de la  $\beta_2$  macroglobuline dans le sang et dans le L.C.R. *Ann. Inst. Pasteur*, 1964, 107, 415-421.

- (96) MATTERN, P., DURET, J., & PAUTRIZEL, R. Hyper- $\beta_2$  - macroglobulinémie au cours de la trypanosomose expérimentale du lapin à Trypanosoma equiperdum. C.R. Acad. Sci (Paris) 1963, 256, 820-822.
- (97) MATTERN, P., KLEIN, F., RADEMA, H., & VAN FURTH, R. Les  $\gamma$ -macroglobulines réactionnelles et paraprotéiniques dans le sérum et dans le liquid céphalo-rachidien humain. Ann Inst. Pasteur, 1967, 113, 857-866.
- (98) MATTERN, P., MASSEYEFF, R., MICHEL, R., & PERETTI, P. Etude immunochimique de la  $\beta_2$  macroglobuline des sérums de malades atteints de trypanosomiase africaine à T. gambiense. Ann Inst. Pasteur, 1961, 101, 382-388.
- (99) McNEILLAGE, G.J.C., HERBERT, W.J. & LUMSDEN, W.H.R., Antigenic type of first relapse variants arising from a strain of Trypanosoma (Trypanozoon) brucei. Exp. Parasitol., 1969, 25, 1-7.
- (100) MICHAELI, D., & FUDENBERG, H.H. The incidence and antigenic specificity of antibodies against denatured human collagen in rheumatoid arthritis. Clin. Immunol. Immunopath., 1974, 2, 153-159.
- (101) MILLER, F., & METZGER, H., Characterization of a Human macroglobulin. I. The Molecular weight of its subunit. J. Biol. Chem. 1965, 240, 3325-3333.
- (102) MURRAY, P.K., JENNINGS, F.W., MURRAY, M., & URQUHART, G.M. The nature of immunosuppression in Trypanosoma brucei infections in mice. I. The role of the macrophage. Immunology, 1974a, 27, 815-824.
- (103) MURRAY, P.K., JENNINGS, F.W., MURRAY, M. & URQUHART, G.M. The nature of immunosuppression in Trypanosome brucei infections in mice. II. The role of the T and B lymphocytes. Immunology., 1974b, 27, 825-840.
- (104) MURRAY, P.K., URQUHART, G.M., MURRAY, M., & JENNINGS, F.W. The response of mice infected with T. brucei to the administration of sheep erythrocytes. Trans R. Soc. Trop. Med. Hyg. 1973, 67, 267.

- (105) MUSCHEL, L.H., SIMONTON, L.A., WELLS, P.A. & FIFE, E.H.J.R., Occurrence of complement fixing antibodies reactive with normal tissue constituents in normal and disease states. J. clin. Invest., 1961, 40, 517-524.
- (106) NIELSEN, K. Preparation of Antisera to the  $\mu$  chain of IgM. J. Immunol. Methods, 1976, 11, 77-82.
- (107) NJOGU A.R. & HUMPHRIES, K.C. The nature of the 4S antigens of the brucei subgroup trypanosomes. Exp, Parasitol., 1972, 31, 178-187.
- (108) NORMANSELL, D.E. Anti  $\gamma$ - globulins in rheumatoid arthritis sera - I. Studies on the 22 S complex. Immunochemistry, 1970, 7, 787-797.
- (109) NOWOTNY , A. Basic exercises in immunochemistry, Springer-Verlag, 1969. Berlin, Heidelberg, New York.
- (110) ONOUE, K. KISHIMOTO, T., & YAMAMURA, Y. Papain fragmentation of the subunits of human macroglobulin. J. Immunol., 1967, 98, 303-313.
- (111) PANUSH, R.S. Rheumatoid factors. J. Florida M.A., 1974, 61, 725-727.
- (112) PARKHOUSE, R.M.E. Non-covalent association of IgM subunits produced by reduction and alkylation. Immunology, 1975, 27, 1063-1071.
- (113) PERCHALSKI, J.E., CLEM, L.W., & SMALL, P.A., 7S Gamma-M immunoglobulins in normal human cord serum. Amer. J. Med. Sci., 1968, 256, 107-111.
- (114) POLSON, A. Quantitative Gel precipitin tests. Science Tools, 1958, 5, 17-20.
- (115) POLSON, A. New approaches to ultracentrifugation, in "Methods in virology", Vol V. 1971 (K. Maramorosch & H. Koprowski), p 33-77. Academic Press, New York & London.
- (116) POLSON, A. Personal communication, 1976.

- (117) POLSON, A & CRAMER, R., Zone electrophoresis of type I polyomyelitis virus. *Biochem Biophys. Acta*, 1958, 29, 187-192.
- (118) POLSON, A., POTGIETER, G.M., LARGIER, J.F., MEARS, G.E.F. & JOUBERT, F.J. The fractionation of protein mixtures by linear polymers of high molecular weight. *Biochim. Biophys. Acta*, 1964, 82, 463-475.
- (119) POLSON, A., & RUSSELL, B., Electrophoresis of viruses. in "Methods in Virology II" (ed. K. Maramorosch, H. Koprowski), p. 391-426. Academic Press, New York, London, 1967.
- (120) PUTNAM, F.W. Alpha, beta, gamma, omega - the roster of the plasma proteins, in "The Plasma Proteins", 1975, Vol. I, 2nd edition, (F.W. Putnam, ed), P.57-131. Academic Press, New York.
- (121) RAADT, P. de. Immunity and antigenic variation: clinical observations suggestive of immune phenomena in African trypanosomiasis, in "Trypanosomiasis and Leishmaniasis with special reference to Chaga's disease" 1974, p. 199-224. Ciba Foundation Symposium 20 (new series) Elsevier, Excerpta Medica. North-Holland. s)
- (122) RAADT, P. de, HART, G. GRANGE, E.B., MBWABI, D. & KWEYAMYA. Serological tests in T. rhodesiense infections East Afr. Trypanosomiasis Res. Organ. Rep. 1967, 1966, 39-42.
- (123) RENN, D.W. & EVANS, E., Use of heteropoly acids as immunological precipitin brighteners. *Analyt. Biochem*, 1975, 64, 620-623.
- (124) ROSE, N., & BRINCKERHOFF, C.E. Characteristics of some "natural" autoantibodies in rabbits. *J. Immunol.* 1969, 102, 682-687.
- (125) ROSE, H.M., RAYAN, C., PEARCE, E., & LIPMAN, M.O. Differential agglutination of normal and sensitized sheep erythrocytes by sera of patients with rheumatoid arthritis, *Proc. Soc. Exp. Biol.*, 1948., 68, 1-6.

- (126) ROTHFIELD, N.F., FRANGIONE, B. & FRANKLIN, E.C.,  
Slowly sedimenting mercaptoethanol-resistant anti-nuclear factors related antigenically to M. immunoglobulins ( $\gamma$ -M globulin) in patients with systemic lupus erythematosus. J. Clin. Invest., 1965, 44, 62-72.
- (127) SANDOR, G., KORACH, S., & MATTERN, P.,. 7S Globulin, immunologically identical to 19S Gamma-1 (beta-2)-M-globulin, a new protein of horse serum. Nature, (London), 1964, 204, 795-796.
- (128) SANTOS-BUCH, C.A., & TEIZEIRA, A.R.L. The immunology of experimental Chagas' disease III. Rejection of allogeneic heart cells in vitro. J. exp, Med. 1974, 140, 38-53.
- (129) SCHEIDEGGER, J.J. Une micro-méthode de l'immuno-électrophorèse. Intern. Arch. Allergy. Appl. Immunol. 1955, 7, 103-110.
- (130) SCHULTZE, H.E. & HEREMANS, J.F., Molecular biology of human protein Vol. I Nature & Metabolism of extracellular proteins. Elsevier, Amsterdam. 1966.
- (131) SEED, J.R. Trypanosoma gambiense and T. equiperdum: Characterization of variant specific antigens. Exp. Parasitol, 1972, 31, 98-108.
- (132) SEED, J.R., CORNILLE, R.L., RISBY, E.L., & GAM, A.A., The presence of agglutinating antibody in the IgM immunoglobulin fraction of rabbit antiserum during experimental African trypanosomiasis. Parasitology. 1969, 59, 283-292.
- (133) SEED, J.R., & GAM, A.M. Passive immunity to experimental trypanosomiasis. J. Parasitol., 1966, 52, 1134-1140.
- (134) SEED, J.R., & GAM, A.A., The presence of antibody to a normal rabbit liver antigen in rabbits infected with Trypanosoma gambiense. J. Parasitol. 1967, 53, 946-950.
- (135) SENECA, H., PEER. P. & HAMPAR, B., Active immunization of mice with chagastoxin. Nature, (London) 1966, 209, 309-310.

- (136) SHEK, P.N., & DUBISKI, S. Maternal-foetal transfer of normal IgM in the rabbit. *Immunology*, 1975, 29, 365-369.
- (137) SINGER, J.M., ALTMANN, G., GOLDENBERG, A., & PLOTZ, C.M., The mechanism of particulate carrier reactions with rheumatoid sera. II Sensitizing capacity of various human gamma globulins for latex particles. *Arthritis Rheum.* 1960, 3, 515-521.
- (138) SINGER, J.M. & PLOTZ, C.M., The latex fixation test. I. Application to the serologic diagnosis of rheumatoid arthritis. *Am. J. Med.* 1956, 21, 888-892.
- (139) SOLOMON, A. Molecular heterogeneity of immunoglobulin-M ( $\gamma$ M-globulin). *J. Immunol.* 1969, 102, 496-506.
- (140) SOLOMON, A., & KUNKEL, H.G., A "monoclonal" type, low molecular weight protein related to  $\gamma$ M-macroglobulin. *Am. J. Med.* 1967, 42, 958-967.
- (141) SOLTYS, M.A. Studies on resistance to Trypanosoma congolense, developed by Zebu cattle treated prophylactically with antrycide pro-salt in an enzootic area of East Africa. *Ann. Trop. Med. Parasitol.* 1955, 49, 1-8.
- (142) SOLTYS, M.A., Immunity in trypanosomiasis. V Immunization of animals with dead trypanosomes. *Parasitology*. 1964, 54, 585-591.
- (143) STAGE, D.E., & MANNIK, M. 7S  $\gamma$ M-globulin in Rheumatoid arthritis. Evaluation of its clinical significance. *Arthritis Rheum.* 1971. 14, 440-450.
- (144) STARITA-GERIBALDI, M. & MASSEYEFF, R. Occurrence of slowly sedimenting components with  $\mu$  chain antigenicity in normal adult and cord serum. *Ann. Immunol. (Inst. Pasteur)* 1975, 126 C, 143-157.
- (145) STASTNY, M. & HOREJSI, J. The interaction of acridine dyes with blood plasma proteins. *Clin. Chim. Acta.* 1961. 6, 782-793.
- (146) STEIGER, R.F. Some aspects of the surface coat formation in Trypanosoma brucei. *Acta Trop (Basel)*, 1971, 28, 341-346.

- (147) STEIGER, R.F., On the ultrastructure of Trypanosoma (Trypanozoon) brucei in the course of its life cycle and some related aspects. Acta. Trop. (Basel), 1973, 30, 64-168.
- (148) STOBO, J.D. & TOMASI, T.B. A low molecular weight immunoglobulin antigenically related to 19 S IgM. J. Clin. Invest., 1967, 46, 1329-1337.
- (149) SWEDLUND, H.A., GLEICH, G.J., & CHODIRKER, W.B. Comparison of certain properties of naturally occurring low molecular weight  $\gamma$ M and the  $\gamma$ M monomer derived by reduction and alkylation of 19 S  $\gamma$ M. J. Immunol. 1968, 100, 1296-1303.
- (150) TAKAYANAGI, T. Protection of mouse from trypanosome infection. Jap. J. Parasitol., 1971, 20, 48 (only).
- (151) TAKAYANAGI, T., & ENRIQUEZ, G.L. Effects of the IgG and IgM immunoglobulins in Trypanosoma gambiense infections in mice. J. Parasitol., 1973, 59, 644-647.
- (152) TAKAYANAGI, T., KAMBARA, H., & INOKI, S., Studies on protection against infection of Trypanosoma gambiense. Jap. J. Parasitol., 1969, 18, 686-687.
- (153) TAKAYANAGI, T., & NAKATAKE, Y. Trypanosome gambiense: immunity with thymic cell transfer in mice. Bxp. Parasitol., 1976, 39, 234-243.
- (154) TERRY, R.J., FREEMAN, J., HUDSON, K.M., & LONGSTAFFE, J.A., Immunoglobulin M. production and immunosuppression in trypanosomiasis: a linking hypothesis. Trans. R. Soc. Trop. Med. Hyg. 1973, 67, 263.
- (155) TIZARD, I.R., & SOLTYS, M.A. Cell mediated hypersensitivity in rabbits infected with Trypanosoma brucei and Trypanosoma rhodesiense. Infect. Immun. 1971, 4, 674-677.
- (156) URQUHART, G.M., MURRAY, M., MURRAY, P.K., JENNINGS, F.W. & BATE, E., Immunosuppression in Trypanosoma brucei infections in rats and mice. Trans. R. Soc. Trop Med. Hyg., 1973, 67, 528-535.



- (157) VAN DALEN, A., SEIJEN, H.G., & GRUBER, M., Isolation and some properties of rabbit IgM immunoglobulin. *Biochim. Biophys. Acts.*, 1967, 147, 421-426.
- (158) VAN REGENMORTEL, M.H.V., Plant virus serology. *Advan. Virus. Res.*, 1966, 12, 207-271.
- (159) VAN REGENMORTEL, M.H.V.; Electrophoresis in "Principles and Techniques in plant virology." C. Kado, & H. Agrawal), P. 390-412. Van Nostrand Reinhold, New York, 1972.
- (160) VAUGHAN, J.H. Behaviour of the rheumatoid arthritis agglutinating factor with immune precipitates. *J. Immunol.*, 1956, 77, 181-188.
- (161) VIENS, P., POULIOT, P. & TARGETT, G.A.T., Cell mediated immunity during the infection of CBA mice with Trypanosoma musculi. *Can. J. Microbiol.*, 1974, 20, 105-106.
- (162) WAALER, E. On the occurrence of a factor in human serum activating the specific agglutination of sheep blood corpuscles. *Acta. Path. Microbiol. Scand.* 1940, 17, 172-188.
- (163) WATSON, R.G. A more specific method for detecting and quantitating rheumatoid factors using a single modification of the RA-test. *Am. J. Clin. Path.*, 1965, 43, 152-156.
- (164) WEEKE, B. Rocket immunoelectrophoresis in "A Manual of Quantitative Electrophoresis". (N.H. Axelsen, J. Krøll and B. Weeke). *Scand. J. Immunol*, 1973a, 2, Suppl. 1. P. 37-46.
- (165) WEEKE, B. Crossed immunoelectrophoresis in "A manual of quantitative immunoelectrophoresis." (N.H. Axelson, J. Krøll, & B. Weeke.) *Scan. J. Immunol.*, 1973b, 2, Suppl. I, P. 47-56.
- (166) WEIR, D.M. Handbook of experimental immunology. 2nd Edition. Blackwell Scientific Publications, Oxford. 1973.

- (167) WHITE, A., Trypanosomatid lipids. Ph.D. thesis, Graduate School of the State University of New York. 1972.
- (168) WHITESIDE, E.F., Interactions between drugs, trypanosome and cattle in the field, in "Drugs, Parasites and Hosts" (L.G. Goodwin, R.H. Nimmo-Smith), 1962. Churchill, London.
- (169) WILLIAMS, J.S., DUXBURY, R.E., ANDERSON, R.I., & SADUN, E.H., Fluorescent antibody reactions in Trypanosoma rhodesiense and T. gambiense in experimental animals. J. Parasitol, 1963, 49, 380-384.
- (170) WILLIAMS, R.C., Rheumatoid arthritis as a systemic disease. Vol. IV in series "Major Problems in Internal Medicine." 1974. p. 154-176. Saunders Company.
- (171) WINBLAD, S., HANSEN, A., & JENSEN, R., The reaction of rheumatoid factor with various animal IgG globulins. Acta. Rheum. Scand., 1969, 15, 200-205.